



BLOOD





# BLOOD

*The Journal of Hematology*

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VOLUME IV, 1949



GRUNE & STRATTON  
NEW YORK

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JOHNSON REPRINT CORPORATION  
NEW YORK, NEW YORK

**First reprinting, 1960, Johnson Reprint Corporation**

VOLUME IV, 1949

# BLOOD

## *The Journal of Hematology*

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## *Contributors to this Issue*

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ALFRED GELLHORN, M D

Associate Professor, Department of Cancer Research, College of Physicians and Surgeons, Columbia University, New York.

SAMUEL KANTOR, M D

Assistant in Medicine, Albany Medical College, Union University, Clinical Assistant in Medicine, Albany Hospital, Albany, N Y

L WHITTINGTON GORHAM, M D

Professor of Medicine and Director of the Department, Albany Medical College, Union University, Physician in Chief, Albany Hospital, Albany, N Y

LINUS PAULING, Ph D

Professor of Chemistry, California Institute of Technology, Pasadena, Calif

SIMON PROPP, M D

Assistant Professor of Medicine, Albany Medical College, Union University, Albany, N Y

J G HUMBLE, M R C S, L R C P

The John Burford Carhill Pathological Laboratories, Westminster Hospital School of Medicine, London, England.

MAX WACHSTEIN, M D

Pathologist, St. Catherine's Hospital, Brooklyn  
Research Assistant, Division of Pathology, Mt Sinai Hospital, New York, N Y

HARVEY A ITANO, M D

National Institute of Health, Postdoctorate Research Fellow, California Institute of Technology, Pasadena, Calif American Chemical Society Predoctoral Fellow

IRVING B WEXLER, M D

Clinical Assistant in Pediatrics, Jewish Hospital of Brooklyn, Associate Transfusionist, Adelpi Hospital, Brooklyn, N Y

LOGAN O JONES, M D

Research Associate, Department of Pharmacology, College of Physicians and Surgeons, Columbia University, New York.

ALEXANDER S WIENER, M D

Senior Bacteriologist (Serology) to the Office of the Chief Medical Examiner of New York City, Chief Transfusionist, Jewish and Adelpi Hospitals, Brooklyn, N Y

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# BLOOD

## *The Journal of Hematology*

VOL IV, NO 1

JANUARY 1949

### RESULTS OF THERAPY OF ERYTHROBLASTOSIS WITH EXCHANGE TRANSFUSION

By ALEXANDER S WIENER, M D , AND IRVING B WEXLER M D

IN PREVIOUS papers<sup>1 2 3 4</sup> we described the method of treatment of erythroblastosis fetalis with exchange transfusion and presented a few illustrative cases in detail. The purpose of the present paper is to summarize our results in the first 28 cases.

The rationale of the therapy of erythroblastosis by exchange transfusion can be briefly outlined as follows. According to our concept<sup>5 6 7</sup> of the pathogenesis of the disease, in the typical case the Rh-positive erythroblastotic baby is born with its red cells coated with univalent Rh antibodies, derived from the mother during intrauterine life by transplacental filtration. In some cases, it is possible that additional Rh antibodies of the bivalent type (agglutinins) may be milked into the fetal circulation by the uterine contractions occurring during labor. In any event, the antibodies acting on the infant's red cells may cause them to hemolyze or to clump (by agglutination or conglutination). In cases in which only hemolysis occurs, a hemolytic anemia results which responds to simple transfusions of Rh-negative blood. If intravascular clumping takes place, on the other hand, the circulation to vital organs may become compromised producing the picture of icterus gravis, often terminating with the death of the infant with the postmortem findings of nuclear jaundice and hepatic necrosis. Obviously, such cases will not be benefited by simple transfusion since such therapy cannot reverse the process of red cell clumping. Luckily, intravascular clumping, when it occurs, probably takes place to greatest extent after birth, because *in utero* the conglutinin content of the fetal plasma is low.<sup>8 9 10</sup> We believe that with the birth of the infant, the conglutinin content may rise to a concentration sufficient to cause clumping of the red cells. The clumping, at first, may be thought of as reversible, the red cells behaving as if they were sticky (sludged blood, Knisely<sup>11</sup>), but in untreated cases, it is probable that the clumping eventually becomes firm, blocking the circulation. If, during the early stages of the disease the infant's blood is drained off and simultaneously replaced with type rh blood of a compatible blood group, it is likely that the disease will become aborted, because type rh blood cells cannot be clumped by the Rh antibodies in the baby's body.

From the Blood Transfusion Division and the Department of Pediatrics of the Jewish Hospital of Brooklyn N Y and from the Serological Laboratory of the Office of the Chief Medical Examiner of New York City

Obviously, in doing exchange transfusions the process of bleeding and infusion must be carried out simultaneously. Thus, the operation becomes progressively less efficient, because as it proceeds, more and more of the donor's blood, and less and less of the infant's blood is withdrawn, so that a complete replacement of blood is theoretically impossible. For practical reasons, it was first decided to limit the exchange to 500 cc. of blood, or about twice the infant's blood volume, and thus effectuate an 87 per cent replacement.<sup>1, 2</sup> It was subsequently found that while this was adequate in the great majority of cases, in more severe cases the remaining 13 per cent of the infant's coated red cells apparently clumped instead of lysing and thus nullified the beneficial effects of the procedure. More recently, therefore, we have modified the procedure, particularly in cases with high antibody titers, by using 1,000 cc. of blood and thus effectuating a 98 per cent replacement.<sup>3</sup> In addition, as our experience has increased, other, less vital, changes have been introduced, calculated to simplify and expedite the operation. In the present paper, with but a single exception (case 10b), only those cases are presented in which 500 cc. of blood were used for the exchange transfusion. In a later paper it is intended to present a second series of cases, for comparison, in which 1,000 cc. exchange transfusions were performed.

#### ANTENATAL MANAGEMENT OF CASES

All pregnant women should be screened to determine if they are Rh positive or Rh negative. Grouping and Rh-Hr typing are done on the husband and all living children of those pregnant women found to be Rh negative, and information obtained as to whether the husband, if Rh positive, is homozygous or heterozygous.<sup>4</sup> In certain instances, the husband's parents must be tested to obtain this important information. Since, when the maternal serum contains univalent Rh antibodies, the severity of the disease usually bears a direct relationship to the titer,<sup>7, 10, 13</sup> the maternal serum is tested at intervals throughout the pregnancy for the presence and titer of antibodies by the saline agglutination and albumin-plasma conglutination techniques.<sup>14</sup>

On the basis of information obtained from these studies, decisions can be made regarding the time of delivery of the infant and the treatment to be instituted after birth. Women who show no sensitization will, of course, be permitted to go to term because their infants will not be erythroblastotic. Mildly sensitized\* women are delivered at term and the infant is treated expectantly and watched for the development of anemia, jaundice, or other signs of erythroblastosis. In those cases where moderate sensitization has developed, the infant is delivered about two weeks before term and treated with immediate exchange transfusion, using 500 cc.

\* Since the same sera in the hands of different workers yield different values each worker must determine for himself what values to describe as low moderate high and lethal. In our laboratory based on our experiences described in this paper the following arbitrary limits have served as our guide: low less than 5 units moderate between 5 and 20 units high, between 20 and 50 units, lethal, above 50 units. This applies only to titers of univalent antibodies by the plasma albumin conglutination method, when the saline titration shows no agglutinin to be present. As will be explained later, in the presence of agglutinins the commonly available methods do not permit a clear-cut identification of univalent antibodies. (Cf. however the recent paper of Wiener and Handman<sup>15</sup>)

of donor's blood for the procedure. More severely sensitized women may even be delivered somewhat earlier and the infant treated by immediate exchange transfusion, using about 1,000 cc. of blood. With very high titers the fetus usually fails to survive until the period of viability, and the resultant dead fetuses are permitted to deliver spontaneously or are aborted.

### RH TYPING AND ANTIBODY TESTS

The bloods of all individuals in each family were classified as to blood group and subgroup, M-N type and Rh-Hr type. The blood grouping and M-N tests were done by the well slide agglutination technic while the Rh-Hr tests were done by the tube agglutination technic. The Rh antisera were obtained in part from male Rh negative donors who had been immunized by injections of Rh positive blood and in part from Rh negative mothers of erythroblastotic infants who, after sterilization, were given stimulating doses of Rh positive blood. While the anti-Rh<sub>0</sub> serum used was a pure agglutinating serum, the anti-rh' and the anti-rh" sera had been prepared from sera of specificity anti-Rh<sub>0</sub> and anti-Rh<sub>0'</sub> by the addition of anti-Rh<sub>0</sub> blocking serum. Anti-hr' serum was available from a type Rh<sub>1</sub>Rh<sub>1</sub> woman who had had an erythroblastotic infant and a small amount of anti-hr" serum had been kindly provided by Dr. A. E. Mourant.

The Rh antibody content of the expectant mother's serum was determined when possible at monthly intervals or more frequently according to the indications by the saline agglutination, albumin plasma conglutination, and at times by the blocking technic. For these titrations fresh suspensions of type Rh<sub>1</sub>, type Rh<sub>2</sub>, and type rh cells were prepared from oxalated group O blood which had been freshly drawn from the vein or stored no longer than seventy-two hours in the refrigerator. All suspensions were washed once by centrifuging, decanting the supernatant, and resuspending the cells in fresh saline to produce a 2 per cent suspension in terms of blood sediment. As mentioned in previous papers, the most common error in the titration technic is in preparing the serum dilutions. Improper rinsing results in carrying over, and accounts for the extraordinarily high titers sometimes reported in the literature. The proper precautions to be followed have been described in previous papers and will not be repeated here. The individual titration technics were carried out as follows:

**Agglutination method.** One drop each of progressively doubled dilutions of the maternal serum was transferred to a series of small test tubes (8 mm. diameter) and to each tube was then added a drop of the test blood suspension. The mixtures were shaken and the rack was placed in the water bath or incubator at 37°C. for one hour. The tubes were then gently tilted one by one in order to dislodge the sediment and the reactions were read under the low power of the microscope by placing the entire tube on the stage under the objective.\*

**Albumin-plasma conglutination method.** A duplicate titration was set up as described for the agglutination method. After the one hour incubation period when the cells had completely sedimented, the supernatant fluid was removed as completely as possible with a fine capillary pipet proceeding from the highest serum dilution to the most concentrated. Then to each tube was added a large drop of fresh albumin plasma mixture, prepared by mixing 4 parts of pooled oxalated plasma from Rh positive individuals with 1 part of 25 per cent human albumin or 30 per cent bovine albumin solution. The tubes were then vigorously shaken to resuspend the cells and were reincubated for another hour at body temperature. At the end of this time the tubes were individually shaken, somewhat more vigorously than for the agglutination technic, and the reactions read under the low power of the microscope.

**Blocking technic.** If the agglutination test was negative and the conglutination showed a significantly high titer, tests were usually carried out by the blocking technic. Again the first step was the titration by the saline agglutination method. Then to each tube was added one drop of an anti-Rh<sub>0</sub> agglutinating serum diluted with saline so as to have a titer of about 10 to 20 units. The mixtures were reincubated for one hour at 37°C. and then the tubes were gently shaken, one by one, and the reactions read under the microscope.

\* By removing the lower half of the ordinary low power objective one is left with a weaker objective which gives lower magnification and ample working space into which to place the test tube.



*Interpretation* For the sake of uniformity all readings were taken by the senior author. If he was out in the laboratory when the incubation period was over the racks were placed on the laboratory table at room temperature until his arrival but this did not seem to affect the results materially. During his absence from the laboratory his assistant read the reactions. The reactions were graded as +++ + + + ±, ++, + and -, where +++ represents one large clump of cells, while ++ and +, etc. progressively weaker reactions and - represents a uniform suspension with no clumps. In the agglutination and conglutination titrations the titer (in units) was taken as the reciprocal of the highest dilution giving a one plus reaction. In the blocking test the titer was taken as the reciprocal of the highest dilution causing complete or almost complete inhibition of agglutination. It was found that the freshness of the test cell suspensions had a more striking effect on the titers obtained than did the Rh type or the zygosity. For example with fresh cells the conglutination titer was usually 20 to 40 times as high as the blocking titer, however if the cells were old the conglutination titer would be lower and the blocking titer higher so that this pitfall could be recognized by a reduction in the ratio. When such abnormal results were obtained the tests were repeated with fresh cell suspensions and in this way mistakes were often avoided. The serologic titration method is crude as compared with chemical titrations and has a large intrinsic error. By performing the titrations with two different Rh positive cells (type Rh<sub>1</sub>rh and Rh<sub>2</sub>Rh<sub>2</sub> as a rule) and averaging the results this error was minimized. The titer values listed in our tables thus represent the average of at least two and usually more titrations.

Even with these precautions the results can be considered to have an intrinsic error of about one tube and this fact must be kept in mind when evaluating the significance of apparent titer fluctuations in tests done serially on any given patient's serum. For example, suppose it is desired to determine if the following series of titers shows any significant fluctuation: 17, 33, 40, 19, 36 and 50. The average of these six values is 33 units. A serum that actually has a titer of 33 units could in tests made at different times give titer values ranging from 16 to 66 units due to variations of technic without indicating that there has been any actual fluctuation in the degree of sensitization. On the other hand had the following values been obtained: 17, 33, 40, 19, 96, 80, 120 then one could assume that there had been a true rise in titer after the fourth sample was tested and that this rise was maintained during the last three tests. In any case of doubt the patient was recalled for another titration.

#### TECHNIC OF EXCHANGE TRANSFUSION\*

The exchange transfusion is carried out immediately after birth, using blood from a compatible, nonsensitized type rh donor. No time is wasted in carrying out conglutination or other serological tests, hemoglobin determinations, erythroblast counts, etc., although blood is taken for these studies. In the event, however, that the father is heterozygous the baby is tested in order to be certain that he or she is Rh positive before proceeding. In certain instances it is possible to predict from the groups of the parents what group of blood will be compatible with the infant's blood.<sup>4</sup> In such cases, the blood can be made available before the baby is born. Where this is not possible, nonsensitized type rh donors belonging to groups A, B, AB and O are kept standing by until the infant's blood group is determined.

The infant is immobilized on a circumcision board, and a 20 gage cannula is inserted into the saphenous vein at the ankle. The infusion of blood is started after the injection of 0.2 cc (200 units) of heparin intravenously. A three-way stopcock connects the tubing of the infusion to the cannula and makes it possible to inject medication as needed and to control the speed of the infusion. A period of fifteen minutes is permitted to elapse before the bleeding is started. This wait is important because it allows time for the heparin to exert its maximum effect and at the same

\* A motion picture demonstrating our technic of exchange transfusion is available to medical societies for loan upon application to the authors.

time permits about 50 cc of blood to enter the infant's circulation and produce enough of plethora to make the arteriotomy an easy procedure. The radial artery is exposed through an incision made just above the lower end of the radius, it is cleaned of all adventitial tissue and lifted up on a hemostat, and with a small scalpel a flap is cut into the artery by inserting the blade into the center of the artery and drawing it diagonally outward. The flow of blood is immediate and copious. The blood is collected in one ounce medicine glasses which are emptied into a graduated bottle. The inflow and outflow of blood are measured carefully and the infusion is kept running about 50 cc ahead of the bleeding at all times. This is easily accomplished by using a syringe on the three-way stopcock to inject the blood at an increased rate when necessary. When 250 cc of blood have run in, a second dose of heparin is given intravenously. No further heparin injections are given, so that by the time the procedure is completed the heparin effect is nullified. When an 1,000 cc exchange transfusion is performed the final dose of heparin is given after 500 cc of blood has been injected. A 10 cc syringe containing a 10 per cent calcium gluconate solution is kept on hand at all times during the procedure, and if signs or symptoms of hypocalcemia supervene, 5 cc are injected carefully directly through the cannula. As a rule, no calcium is required for transfusions of 500 cc or less, provided the transfusion is not given too rapidly, that is, within less than an hour. For larger exchange transfusions, it is best to inject 5 cc of calcium gluconate prophylactically at the 500 cc mark, even though the patient exhibits no untoward symptoms. In 500 cc exchange transfusions the amount of blood injected should exceed the amount withdrawn by about 50 cc, in the 1000 cc transfusions a margin of about 75 cc should be allowed. In infants severely anemic at birth this margin should be increased by another 50 cc.<sup>15</sup> At the close of the procedure the radial artery is usually tied off before closing the incision at the wrist, but a snug bandage will control any venous oozing from the incision at the ankle. A small amount of sulfadiazine powder is placed into the wounds before closing them, and the infant is routinely given 20,000 units of penicillin intramuscularly every three hours for twenty-four hours after the operation. Subsequent treatment of the infant is routine, except that breast feeding is interdicted.

### RESULTS

For the purpose of evaluating the efficacy of the treatment, the cases have been divided into four categories as follows:

- (1) Severe cases, with recovery, (2) fatal cases, (3) cases of moderate severity, and (4) mild cases.

#### *Severe Cases, with Recovery*

CASE 1.—This case has been presented in detail elsewhere<sup>2</sup> and to conserve space will not be repeated here. It is however included in the discussion at the end of this paper.

CASE 2.—This patient was referred to us twenty-four hours after birth. He was the product of the second pregnancy, the first and only previous pregnancy having been normal. The mother had never received any blood transfusions. The infant was jaundiced at birth and the splenic edge was palpable 3 fingerbreadths below the costal margin. After several hours an anemia of 8.8 grams per cent of hemoglobin was found with a red blood count of 3.71 million red blood cells per cu mm. The uncorrected

white blood count was 64,000 cells and there were 296 normoblasts per 100 white blood cells on the smear. Anisocytosis and poikilocytosis were present to a marked degree.

*Findings:* Grouping and Rh Hr tests done on the family are shown in table 1.

Antibody studies done on the mother's plasma by the agglutination technic showed the presence of Rh antibodies of a titer of 20 units, while the titer of the Rh antibodies was 30 units by the plasma conglutination test and 70 units by the albumin plasma conglutination technic.\* The conglutination test for coating of the infant's red cells by univalent antibodies was positive.

*Prognosis:* The findings in this case pointed to a serious prognosis, namely the high titer of Rh antibodies in the maternal serum, the infant's deep jaundice, severe anemia, unusually high erythroblastemia as well as its poor clinical condition.

*Procedure:* At the start of the exchange transfusion the infant appeared to be in a condition bordering on shock. A subconjunctival hemorrhage was present in the left eye and small petechiae had appeared on the forehead. The skin had a mottled appearance and there were occasional nystagmoid movements of the eyes. The exchange transfusion was performed using blood from a donor who belonged to type OMNrh. In order to reduce the conglutinin content, the donor's blood was first treated by removing half of the plasma and replacing it with normal saline solution. Of this mixture, 560 cc. were injected while 500 cc. of blood were simultaneously withdrawn.

TABLE 1

Blood of	Group and subgroup	H N type	Rh-Hr type	
			Phenotype	Genotype
Father	B	N	Rh <sub>1</sub> Rh <sub>1</sub>	R <sup>1</sup> R <sup>1</sup> or R <sup>1</sup> r'
Mother	O	MN	rh	rr
1st son	O	MN	Rh <sub>1</sub> rh	R <sup>1</sup> r
Patient	O	MN	Rh <sub>1</sub> rh	R <sup>1</sup> r

\* The M N tests are not clinically important but are included for the sake of completeness.

*Results:* The immediate response was most dramatic. The infant appeared to be more vigorous and although there was no change in the jaundice the color and circulation were definitely improved. The hemoglobin on the day following the transfusion was 11.7 grams per cent and then rose to 13.2 grams. Jaundice became intense by the fifth day at which time the icterus index had risen to 120 units. During this time the baby was irritable and took his feeding poorly. By the sixth day marked clinical improvement was noted. The jaundice began to fade the petechiae noted on the forehead had been completely absorbed and from that time onward the baby acted well. The hemoglobin concentration continued to decrease over a period of a month when it reached a concentration of 6.6 grams per cent with a red blood count of 2.5 million per 100 mm. By this time the icterus had faded completely. The infant was given a transfusion of 70 cc. of O rh blood on the following day and the hemoglobin concentration rose to 9.2 grams. This child has been followed carefully for more than a year. Both his physical and mental progress had shown no deviation from the normal. He sat at five months and stood at 11 months. At one year he was beginning to take his first steps. His first four teeth have a greenish discoloration.

*CASE 3:*—This patient had had five miscarriages at 3 to 5 months over a period of three years. Her sixth pregnancy yielded a full term infant who is normal and well. This child showed no jaundice or anemia during her neonatal period. When first seen by us the mother was in the 32nd week of her seventh pregnancy and had been found to be Rh negative.

*Findings:* Groupings and Rh Hr tests done on the family are shown in table 2.

Antibody tests for Rh sensitization done on the mother's serum at this time showed agglutinins to be present in a titer of 7 units, while the titer was 16 units by the plasma conglutination technic. One month

\* The figures for antibody titers given in this paper represent averages of the results of at least two titrations.<sup>12, 14</sup>

later, the agglutinin titer was still 7 units, while the titer by the plasma conglutination technic was 20 units (This difference is not significant because it is within the limits of accuracy of the method of titration)

**Prognosis** This woman, then, was moderately sensitized to the Rh factor, and since the husband was most likely homozygous for Rb<sub>0</sub>, an Rh<sub>1</sub>rh or Rh rh infant could be expected who would have erythroblastosis in a severe form, and might even be stillborn if carried to term

**Procedure** Labor was induced in the thirty-eighth week of the pregnancy. Plans had been made to do an exchange transfusion immediately after birth, but the baby was born in a city many miles away and nine hours elapsed before we arrived at the hospital. In the meantime the infant had been found to have a hemoglobin of only 9.7 grams per cent and there were 12 normoblasts per 100 white blood cells on the smear. The baby was given a transfusion of packed red cells from 100 cc of O, rh bank blood. Shortly thereafter, the infant became cyanotic and was placed in an incubator. Moderate jaundice as well as cyanosis and difficulty in respiration were present when we saw the child. A few râles were present, scattered throughout both lung fields, which were interpreted as due to atelectasis. Although the transfusion had raised the hemoglobin concentration to 13.6 grams, an exchange transfusion was carried out with the administration of 560 cc of blood (half of the plasma in this blood was replaced by saline solution) and the simultaneous removal of 600 cc of blood. During the procedure the infant required repeated aspiration of mucus and inhalation of oxygen because of several episodes of cyanosis. In tests carried out later, the cord blood of the baby typed as OMRb<sub>2</sub> and the icterus index was 40 units.

TABLE 2

Blood of	Group and subgroup	M N type	Rh Hr type	
			Phenotype	Genotype
Father	O	M	Rh <sub>1</sub> Rh <sub>2</sub>	R <sup>1</sup> R <sup>2</sup> or R <sup>1</sup> r <sup>2</sup>
Mother	O	M	rh	rr
Daughter	O	M	Rb <sub>1</sub> rh	R <sup>1</sup> r

**Results** Differential agglutination studies showed that the combined procedures had left only 2 per cent of the infant's own blood cells in its circulation. The hemoglobin concentration of the blood was 11.6 grams per cent on the day following the transfusion and the child was clinically very much improved. The infant remained well, but the hemoglobin concentration fell gradually and on the fourteenth day of life another transfusion of 75 cc of Rh negative blood was given. From this point on the baby did very well. This child has been followed with great care for about a year and has been unusually healthy as well as having showed rather precocious advancement from the developmental point of view.

CASE 4—This case has already been reported in detail elsewhere.<sup>4</sup>

CASE 5—The mother of this patient was first seen by us in the twelfth week of her third pregnancy. Her first pregnancy terminated with the birth of a normal female who is well. Her second pregnancy was attended by a midwife, labor lasted two days and yielded an apparently normal infant who was jaundiced for a few days and then seemed to recover. This baby was nursed for several days during the neonatal period. At the age of seven months the child was unable to hold up its head, had athetoid movements, followed light poorly, and had a vacuous expression.

**Findings** Grouping and Rh Hr tests done on the father, mother and both children are shown in table 3.

Antibody studies done on the mother's serum at intervals during her pregnancy are given in table 4.

At the time of the last test, titrations for alpha and beta antibodies were done since a possibility of double sensitization (to A as well as to Rh) existed. By the agglutination technic the anti A titer\* was 48 units while the Anti B titer was also 48 units. With the albumin plasma method the anti A titer was 160 units and the anti B titer was 48 units.

\* Using test cells of subgroup A.

*Prognosis* On the basis of these findings there appeared to be little doubt that the expected infant would be severely affected by the disease and might even be stillborn if the pregnancy were allowed to go to term. In addition to the harm that would be done by the univalent Rh antibodies, one might expect some injury to be caused by the alpha antibodies that were present if the baby proved to be group A.

*Procedure* Delivery was spontaneous at term. The infant appeared normal at birth and had a hemoglobin concentration of 15.5 grams per cent. The erythrocytes typed as  $A_2MNRh_1rh$  and were shown by the conglutination technic to be coated with univalent antibodies. Immediate exchange transfusion was carried out using blood from a group A, type rh donor from which one-half of the plasma had been removed and replaced with saline. Over a period of 90 minutes 500 cc. of blood were administered and 450 cc. removed.

*Results* The infant withstood the procedure well. The hemoglobin concentration of the blood after the transfusion was 16 grams per cent. However, by the seventh day it had fallen to 13.5 grams per cent and the patient became severely jaundiced. On the eighth day of life the serum bilirubin concentration was 16 mg. per cent, but this fell to normal by the fifteenth day. During this time there was a gradual

TABLE 3

Blood of	Group and subgroup	M N type	Rh Hr type	
			Phenotype	Genotype
Father	$A_2$	MN	$Rh_1rh$	$R^1R^0$ or $r^1R^0$
Mother	O	M	rh	rr
1st child	O	M	$Rh_0$	$R^0r$
2nd child	O	M	$Rh_0$	$R^0r$

TABLE 4

Week of pregnancy	Titer by agglutination technic (units)	Titer by plasma conglutination technic (units)	Titer by albumin plasma conglutination technic (units)
12th week	0	6	—
20th week	0	$\frac{1}{2}$	—
28th week	0	$1\frac{1}{2}$	$5\frac{1}{2}$
34th week	0	2	12
37th week	0	6	44

decline in the hemoglobin concentration of the blood to 8.5 grams per cent and the baby was given another transfusion this time of Orh blood on the fifteenth day, following which the hemoglobin concentration rose to 15.5 grams. Except for a bout of diarrhea that developed at the age of one month the baby has since done well.

This case is instructive in illustrating the method of determining genotypes. The father belonged to phenotype  $Rh_1rh$  so that on this basis he belonged to one of the three genotypes,  $R^1r$ ,  $R^0r^1$ , or  $R^1R^0$  of which the first is the most common and therefore the most likely. For this reason, type  $Rh_1rh$  individuals are usually presumed to be heterozygous.<sup>8</sup> However, when it was found that the first two children belonged to type  $Rh_0$ , this excluded genotype  $R^1r$  leaving genotypes  $R^1R^0$  and  $R^0r^1$  as the remaining possibilities. When finally the new baby proved to be  $Rh_1$  it was apparent that the genotype of the father is  $R^1R^0$  so that he is homozygous for the  $Rh_1$  factor even though he belongs to phenotype  $Rh_1rh$ . Obviously, every future child of this couple will be erythroblastotic.

This case is unusual in that the baby developed a hemolytic anemia despite the exchange transfusion and required a supplementary transfusion before the blood count was stabilized. It is possible that this was due to sensitization with the A factor, and this interpretation is supported by the excellent response to subsequent transfusion of group O, type rh blood.

Despite the additional complication of diarrhea, the child did well, is one year old at the time of this writing and is normal.

**CASE 6**—This baby was referred to us at the age of 1 day for treatment by exchange transfusion. Studies done elsewhere had shown the mother to be Rh negative and the father to be Rh positive. Blocking antibodies were detected in the mother's serum during her pregnancy and were said to be 4 plus. The baby was the result of the second pregnancy. The first baby was jaundiced at birth but the jaundice cleared after several days without treatment and this child is well. One and one half years before the onset of the first pregnancy the mother had received a transfusion of blood from her husband.

**Findings:** On examination the infant was pale and jaundiced. The hemoglobin concentration of the blood was 11.4 grams. The spleen and liver were moderately enlarged and the child's general condition was good.

Grouping and Rh-Hr tests done on the father, mother and the infant are shown in table 5.

No antibodies could be detected by the saline agglutination test in the mother's serum but the blocking test<sup>17</sup> was positive to a titer of 1½ units. By the albumin plasma technic, univalent Rh antibodies

TABLE 5

Blood of	Group and subgroup	M N type	Rh-Hr type	
			Phenotype	Genotype
Father	O	N	Rh <sub>1</sub> Rh <sub>1</sub>	R <sup>1</sup> R <sup>1</sup> or R <sup>1</sup> r'
Mother	A <sub>1</sub>	M	rh	rr
Infant	O	MN	Rh <sub>1</sub> rh	R <sup>1</sup> r

were demonstrable in a titer of 40 units. Furthermore, the infant's cells were completely coated\* by univalent Rh<sub>0</sub> antibodies as shown by the fact that they behaved in the tests as though they belonged to type rh.<sup>18</sup> In addition, free univalent antibodies were demonstrated in the baby's serum in a titer of 3 units by the albumin plasma agglutination test.

**Prognosis:** This infant was already erythroblastotic when seen and in view of the complete coating of the erythrocytes by univalent antibodies was probably in imminent danger of developing serious intra-vascular clumping.

**Procedure:** Exchange transfusion was performed using the blood of a donor belonging to group O type rh. One half of the donor's plasma was removed and replaced with normal saline in order to reduce the conglutinin content of the infused blood. Five hundred and fifty cc. were administered into the saphenous vein and 500 cc. of blood were withdrawn from the radial artery. The baby was returned to the ward in excellent condition.

**Results:** The day following the procedure the hemoglobin concentration of the blood was 12 grams per cent and the infant's general condition was good. The icterus index which had been 60 units at the onset of the procedure, was now 64 units and differential agglutination tests showed that a replacement of about 85 per cent of the red cells had been accomplished.

Two days after the transfusion the baby began to show signs of irritability and his temperature rose to

\* The cells of all typical erythroblastotic babies are coated by univalent antibodies as can be demonstrated by suspending the cells in plasma or albumin plasma mixture or by the anti globulin technic. If in addition the baby's Rh positive cells are 'blocked' as shown by their failure to clump in good anti Rh<sub>0</sub> agglutinating serum they are considered to be completely coated.

101 F The urine was found to contain 10-25 white blood cells per high power field and culture was positive for staphylococcus aureus. The Chvostek, peroneal and Trousseau signs were positive and the serum calcium was found to be only 7.3 mg per cent. The child was treated with intravenous calcium gluconate, calcium by mouth and given penicillin and sulfa therapy. Within a few days the temperature fell to normal, the calcium concentration of the serum returned to normal levels and the urine cleared. When discharged at the end of eight days, the child's hemoglobin concentration of the blood was 10.6 grams and he was clinically well. At the age of 3 months the child weighed 15 pounds and the hemoglobin concentration was 12.2 grams per cent. There were no subsequent transfusions given, and the child developed normally.

CASE 7—The mother of this infant was first seen in the eleventh week of her fourth pregnancy. Her first and second pregnancies had ended at term, and both of these children are alive and well. Her third pregnancy resulted in the birth of a full term infant who seemed to be well at first but then became

TABLE 6

Blood of	Group and subgroup	M-N type	Rh Hr type	
			Phenotype	Genotype
Father	O	M	Rh <sub>1</sub> Rh	R <sup>1</sup> R <sup>1</sup> or R <sup>1</sup> r
Mother	A <sub>1</sub>	M	rh	rr
1st daughter	O	M	Rh <sub>1</sub> rh	R <sup>1</sup> r
2nd daughter	A <sub>1</sub>	M	Rh <sub>1</sub> rh	R <sup>1</sup> r

TABLE 7

Week of pregnancy	Titer by agglutination technic (units)	Titer by plasma conglutination technic (units)	Titer by albumin-plasma conglutination technic (units)
11th week	0	15	—
10th week	0	10	—
32nd week	0	4	12
38th week	0	11	30

jaundiced and was transfused eight hours after birth. This infant died on the third day of life. The clinical diagnosis made at that time was cerebral hemorrhage.

*Findings.* Grouping and Rh Hr tests done on the father, mother and both surviving daughters gave the results shown in table 6.

Antibody studies done on the mother's blood at intervals during her pregnancy gave the results shown in table 7.

*Prognosis.* An autopsy report on the infant that died is not available but from the clinical symptoms described it is evident that the death may have been due to erythroblastosis. This belief is strengthened by the finding of a moderately high antibody titer in the maternal serum early in the following pregnancy. Since the father was Rh positive and almost surely homozygous it was anticipated that the new baby would be Rh positive and also have moderately severe erythroblastosis.

*Procedure.* In order to limit the period of time over which the infant would be exposed to the action of the maternal antibodies, labor was induced two weeks before term. The infant, a girl, appeared to be normal. There was a faint yellow streak along the umbilical cord but the amniotic fluid was not yellow and the vernix was not discolored. Exchange transfusion was carried out immediately using 540 cc. of blood from an A<sub>1</sub>Nth donor for the infusion while 480 cc. of blood was withdrawn. Half of the plasma had been removed from the donor's blood and replaced with saline in order to reduce the conglutinin content of the infused blood. The baby withstood the procedure well.

**Results** The infant's course was entirely uneventful except for the appearance of moderate jaundice on the second day. This subsided rapidly. No hepatic or splenic enlargement was made out at any time. The infant's blood group was  $A_1MRh_{17}rb$ , and a positive reaction was obtained with the agglutination test for coating of the infant's red cells. Furthermore, free Rh antibodies of 4 units titer could be demonstrated in the baby's serum by the agglutination method. On the day following the transfusion the hemoglobin was 17.4 grams per cent and the red blood cell count 5.04 million per cu mm. Three normoblasts per 100 white blood cells were present on the smear. The child left the hospital on the fifth day of life in excellent condition. When seen again at the age of 4 months the child was alert and held its head up well.

**CASE 8**—This baby, a female, was first seen on the second day of life. No antenatal studies had been done on the mother during pregnancy. Her first child was born two years previously and was well. The mother had received no transfusions or blood injections at any time. At birth the infant appeared to be normal but on the second day of life rapidly became jaundiced. The hemoglobin concentration was found to be 11.7 grams per cent and the red blood count 2.9 million per cu mm. Seven nucleated red blood cells per 100 white blood cells were found on the smear.

**Findings** Grouping and Rh Hr tests done on the father, mother, and infant gave the results shown in table 8.

Weak agglutinins were demonstrable in a titer of 8 units in the mother's serum but by the albumin plasma agglutination technique univalent antibodies were demonstrable in a titer of 40 units.

TABLE 8

Blood of	Group and subgroup	M N type	Rh Hr type	
			Phenotype	Genotype
Father	A	M	Rb <sub>1</sub> rh	R <sup>1</sup> R <sup>0</sup> or R <sup>0</sup> r'
Mother	A <sub>1</sub>	MN	rb	rr
Patient	O	M	Rb <sub>0</sub>	R <sup>0</sup> r

**Prognosis** Inasmuch as the above titers were determined only one day postpartum, these titers were presumably the same as those existing just before delivery, so that moderately severe sensitization was present and the prognosis was fair if intravascular clumping had not already occurred, and provided that an exchange transfusion were done immediately. With simple transfusion therapy the likelihood of recovery seemed to be remote.

**Procedure** Exchange transfusion was performed when the baby was 36 hours old. Over a period of one hour 550 cc. of group O, type rh blood were injected and 475 cc. of blood removed.

**Results** The hemoglobin concentration of the blood was 15.9 grams per cent after the transfusion with a red blood count of 5.2 million per cu mm. There were 4 nucleated red blood cells per 100 white blood cells on the smear. The icterus index before the procedure was 70 units and after the procedure had been reduced to 50 units. Titration of free Rh antibodies by the albumin plasma agglutination method done on the infant's plasma showed a pretransfusion concentration of 1½ units and a post transfusion concentration of 1½ units.

The baby withstood the procedure very well and the jaundice had almost completely faded three days after treatment, at which time the baby was discharged from the hospital. When seen at the age of five weeks the hemoglobin concentration was 14.3 grams per cent and the red blood cell count was 5.05 million per cu mm. the baby was entirely well clinically.

The obstetrician was so impressed by the improvement of the baby by the transfusion that he sent her home with her mother on the fourth day postpartum without even consulting us. This spectacular result cannot be duplicated by any case seen in the days before exchange transfusion. Comparable cases in the past have



either showed progressive jaundice despite transfusion, with early death from kernicterus, or have recovered following a series of transfusions over a period of weeks or months, sometimes only to exhibit sequelae of liver and brain damage later on

**CASE 9**—When first seen, the mother of this patient was in the interval between her first and second pregnancy. Her first pregnancy, three months previously, had been terminated by cesarean section in the thirty fifth week because of central placenta previa. She received two transfusions at that time. The infant weighed 2½ pounds and lived for only twelve hours. Studies were requested to determine if isoimmunization had any bearing on the loss of the infant.

**Findings** Grouping and Rh Hr tests done on the husband and wife gave the results shown in table 9.

At the time that these studies were done tests for antibodies in the mother's serum showed an agglutinin titer of 2 units while the titer by the plasma conglutination test was also 2 units. It seemed much less likely that the sensitization had been caused by the pregnancy than by the two transfusions that the woman had received. Inasmuch as the husband was most probably heterozygous for the Rh factor there

TABLE 9

Blood of	Group and subgroup	M-N type	Rh Hr type	
			Phenotype	Genotype
Husband	A <sub>1</sub>	M	Rh <sub>1</sub> rh	R <sup>+</sup> R <sup>+</sup> , or r'R <sup>+</sup>
Wife	A <sub>1</sub>	M	rh	rr

TABLE 10

Week of pregnancy	Titer by agglutination technic	Titer by albumin plasma technic
24th week	0	0
32nd week	9	13
33th week	2½	4½
34th week	10	6
35th week	7	15
37th week	6	10

was an even chance that any future pregnancy would produce either an Rh positive or an Rh negative infant. Furthermore, since sensitization was only mild there was a possibility that even if she had an Rh positive infant it would be only moderately or mildly affected and could be saved by the proper treatment.

The mother returned fourteen months later for further studies, in the twenty fourth week of her second pregnancy, and her serum was tested for antibodies at frequent intervals thereafter with the results shown in table 10.

**Prognosis** The absence of antibodies at the first examination followed by their appearance at the second examination, indicated that the mother was carrying an Rh positive fetus and that a moderately affected erythroblastic infant could be expected.

**Procedure** Because of the previous cesarean section it was felt that this child should also be delivered transabdominally. In order to limit the period of time that the infant would be in contact with the maternal antibodies cesarean section was done at thirty-seven weeks. Before proceeding with the exchange transfusion however the baby's blood was grouped and Rh tested in order to be certain that we were not dealing with an Rh negative child. As expected from the antibody tests the baby was Rh positive (A<sub>1</sub>MRhrh).

Exchange transfusion was performed using blood from an A<sub>1</sub>MNrh donor. Over a period of ninety minutes, 550 cc. were given and 500 cc. removed. The baby bore the procedure well.

*Results* The hemoglobin concentration at birth was 17.4 grams per cent, and the red blood count 6 million per cu mm. There was only 1 normoblast per 100 white blood cells on the smear. The icterus index was 28 units. The hemoglobin concentration following the procedure was 13.1 grams per cent and the red blood count 4.8 million per cu mm. The albumin plasma conglutination test on the cord serum could detect no free Rh antibodies, but by the acacia method a titer of 48 units was obtained on the baby's serum.\*

On the day following the transfusion jaundice appeared and deepened perceptibly on the second day.† The icterus index at this time had risen to 72 units. The infant remained clinically well, however, and took its feedings without difficulty. The hemoglobin concentration remained unchanged. The spleen became slightly enlarged, but the liver was not palpable. By the eighth day the spleen was no longer palpable and the jaundice was fading rapidly. The patient was discharged from the hospital at the age of 2 weeks. At the age of 2 months the hemoglobin concentration had fallen to 8.4 grams, but reticulocytes were present on the blood smear and a differential agglutination test showed that as much as 90 per cent of the infant's blood was Rh positive, indicating that regeneration of blood was proceeding at a satisfactory rate, and that further transfusion was not necessary.

When seen again at the age of 3 months, the child was perfectly well and developing normally both mentally and physically.

TABLE II

Blood of	Group	M N type	Rh Hr type	
			Phenotype	Genotype
Father	O	MN	R <sub>h</sub> 1R <sub>h</sub> 1	R <sup>1</sup> R <sup>1</sup> or R <sup>1</sup> r <sup>1</sup>
Mother	O	M	rh	rr
1st son	O	M	R <sub>h</sub> 1rh	R <sup>1</sup> r
2nd son	O	MN	R <sub>h</sub> 1rh	R <sup>1</sup> r

### Fatal Cases

CASE 101.—The mother of this patient was first seen in the second trimester of her fifth pregnancy. Her first two pregnancies had yielded normal infants who are well today. Her third pregnancy resulted in the birth of a stillborn fetus at term. Life had been felt until three days before delivery. Her fourth pregnancy terminated spontaneously at 36 weeks with the birth of a stillborn infant which had apparently been dead *in utero* for about two weeks.

*Findings* Grouping and Rh Hr tests done on the entire family gave the results shown in table II.

Antibody tests done on the mother's plasma during the second trimester were positive to a titer of 4 units both in saline and plasma media, indicating a mild sensitization to the Rh factor with antibodies predominately of the bivalent type. At the beginning of the third trimester, however, the antibody titer had risen to 12 units by the agglutination method and to 25 units by the plasma conglutination technique.

*Prognosis* Since the husband was presumably homozygous for the Rh factor there was little doubt that the stillborn infants from the third and fourth pregnancies had died of erythroblastosis. Furthermore, in view of the significant rise in antibody titer, the expected infant would undoubtedly be Rh positive and stillborn if the pregnancy were allowed to go to term.

*Procedure* A male infant weighing 5 lbs., 6 oz. was delivered by cesarean section six weeks before term. At birth the cord was seen to be bile stained and the infant was pale and had a weak cry. Blood was taken

\* That this was not an artefact was proved by demonstrating that it was possible to distinguish Rh positive and Rh negative bloods by the acacia conglutination method using the infant's serum diluted 1 to 8 with saline solution as the testing serum.

† Subsequent experience has shown that it is not uncommon for jaundice to increase for a day or two after the exchange transfusion before subsiding. This may be due to liver damage sustained before the institution of treatment.

for subsequent examination, but the exchange transfusion was carried out without delay. A total of 420 cc of blood was withdrawn and 500 cc injected. The baby withstood the procedure well. Subsequent tests showed that at birth, the hemoglobin concentration was 11.7 grams per 100 cc, the red blood cell count 2.62 million per cu mm with 18 nucleated red blood cells per 100 white cells on the smear. The total white blood count was 23,200 per cu mm. The icterus index at birth was 70 units. As expected the baby was Rh positive, the complete classification being OMNRh<sub>1</sub>rh<sup>+</sup>.

**Results.** Following the transfusion the hemoglobin concentration was 19.8 grams per cent, and differential agglutination showed that an exchange of about 90 per cent had been accomplished. The icterus deepened on the second day of life although the baby seemed to be clinically well. There was no hepatomegaly or splenomegaly. On the third day of life the baby became intensely jaundiced. The icterus index had risen to 110 units and the infant became lethargic and refused its feedings. In the latter part of the day, brown edema of both lower extremities became evident. The hemoglobin concentration had now fallen to 14.7 grams per cent. The subsequent course was downhill. The temperature fell to subnormal levels, the baby refused feedings and became dehydrated. Despite intravenous fluids he failed to improve. On the fifth day of life the temperature rose to 103.5 F and death ensued.

**Autopsy report.** Kernicterus, hepatosplenomegaly, necrosis of Hassell's corpuscles, hemorrhage into lungs, large areas of necrosis in the liver, islands of hematopoiesis in the liver, spleen and adrenals.

As is usual in many cases of erythroblastosis, the condition of this infant appeared to be excellent at the time of birth and immediately thereafter. We expected, therefore, that if the progress of the disease could be arrested by exchange transfusion this infant would survive. The death of this baby led us, in subsequent cases, to remove half of the plasma from donor's blood and replace it with saline, thus reducing the conglutinin content of the infused material and favoring hemolysis instead of clumping. The promise of this procedure was not fulfilled and it has therefore been abandoned. We have now further changed our procedure by using 1,000 cc of blood instead of 500 cc in the more severe cases, and our limited experience up to the present time indicates that many more of these severe cases can be saved with this modification, which ensures an exchange of 98 per cent of the infant's blood instead of only 87 per cent, thus obviating any possibility of further clumping or hemolysis.<sup>1, 2</sup> Even this modification is not universally successful as will be seen from the case presented below.

**CASE 106.**—This infant was the sibling of case 102. The mother became pregnant again about six months after delivering the baby just described and her blood was carefully followed with repeated antibody titrations prior to delivery. The results of these tests are shown in table 12.

**Prognosis.** In view of the rising titer of antibodies and the history of the loss of three previous infants from erythroblastosis, the prognosis for the expected child appeared to be hopeless if the pregnancy were permitted to go to term; in fact, the fetus would be expected to die *in utero* before the end of the eighth month. The only chance for survival was to deliver the baby while it was still alive and perform a massive exchange transfusion immediately after birth. Even at this time the manifestations were apt to be severe, so that the prognosis was grave.

**Procedure.** Cesarean section was performed six weeks before term. On exposing the uterus a small herniation the size of a walnut was found in the anterior uterine wall. This was covered only with peritoneum and was filled with blood.† On palpation, the hernia ruptured and bled profusely and the

\* Actually, the cells failed to clump in anti Rh<sub>0</sub> serum, due to coating of the red cells by blocking antibodies.

† In view of this defect in the uterine wall, the patient might have died of a ruptured uterus had she been permitted to go into labor. The development of the defect with the resulting detachment of the underlying placenta may account for the rise in the maternal antibody titer, as such a defect would permit fetal blood to enter the maternal circulation and stimulate the production of additional antibodies.

operation was completed rapidly by extending the incision through the herniation. The infant on delivery, weighed 5 pounds and 1 ounce and exhibited extreme pallor. Respirations were shallow and infrequent and a moderately large amount of blood and mucus had to be aspirated from the pharynx and trachea. Before the cesarean operation two donors belonging to group O type rh had been bled of 500 cc each and this blood was ready for immediate transfusion. Within a few minutes the baby was given 100 cc of blood and showed marked improvement in its general condition. It did, however, become cyanotic when oxygen was withheld. Exchange transfusion was then completed with the administration of a total of 1,000 cc of blood and the removal of 950 cc. Throughout the procedure the baby required frequent aspiration and continuous oxygen inhalation. Fifteen cc of 10 per cent calcium gluconate were given in divided doses of 5 cc each during the procedure which took a total of two hours. On being returned to her incubator the infant appeared to be quite well.

**Results:** During the next twenty four hours the baby was fairly active. She had one period of apnea which responded to artificial respiration and she also exhibited a few tremors which responded to the intravenous administration of calcium gluconate. On the morning following the procedure when the baby was about 24 hours old she suddenly expired.

Laboratory studies done on the cord blood obtained at birth showed hemoglobin concentration, 5.8 grams per cent, red blood cells, 1.5 million per cu mm, white blood cells 4,800 per cu mm, polys 33, myelocytes, 2, lymphocytes, 61, monocytes 3, eosinophiles 1. There were 45 nucleated red blood cells

TABLE 12

Week of pregnancy	Agglutinin titer	Titer by albumin plasma conglutination
2 days after 1st menstrual period	4	15
11 weeks	6	36
14 weeks	1	18
23 weeks	0	9
28 weeks	0	6
32 weeks	4	16
34 weeks (4 days before Cesarean operation)	32	26

per 100 white blood cells on the smear. The icterus index was 52 units. The albumin plasma conglutination test for coating of the infant's cells was positive. The baby's group was OMRh<sup>+</sup>.

After the transfusion the hemoglobin concentration of the infant's blood was 13.2 grams per 100 cc and there were 124 nucleated red blood cells per 100 white blood cells.

At autopsy the liver was found to be greatly enlarged with large areas of necrosis, so that there is little doubt that this infant died as the result of damage caused by the maternal antibodies while the fetus was still *in utero*. It may still be possible, we feel, to save some severely affected infants with less involvement of the liver but who would otherwise die, if the hemoglobin concentration is maintained at normal levels by allowing a larger margin of infused blood over that removed. In this case, a margin of only 50 cc was allowed and the hemoglobin concentration was only 13.2 grams per cent following the transfusion. We have subsequently found that when a 100 cc margin instead of 50 cc is allowed in an exchange transfusion of 1,000 cc one is more likely to attain a normal hemoglobin concentration of the newborn, namely, about 16 grams. When the patient is extremely anemic as this infant was, a margin as great as 150 cc is desirable in order to correct the reduction of blood volume usually present in such cases.<sup>15</sup>

CASE 11. This infant was born in a city 250 miles away and was not under our complete management at any time. The only data available to us antenatally was that the mother was Rh negative and we were told that her serum contained Rh agglutinins in a titer of 64 units shortly before birth. The baby became extremely jaundiced by the fourth day of life at this time it was lethargic and cyanotic, and had to be placed in an oxygen tent. Exchange transfusion was then performed by us at the request of the attending physician, as a measure of last resort. The infant seemed to be improved immediately following the procedure, but died about five hours following its completion.

CASE 12. The mother of this patient was first seen by us two years after her fourth pregnancy. Her obstetrical history at that time was as follows. Her first pregnancy terminated with the birth of a male infant who was cyanotic, required resuscitation and lived for only two days. Her second pregnancy yielded a normal male infant who is alive and well. Following this she gave birth to a full term male infant who developed jaundice and lived for only twenty-five hours. Her fourth pregnancy yielded a stillbirth two weeks before term.

*Findings.* Grouping and Rh-Hr tests done on the father, mother and living child gave the results shown in table 13.

TABLE 13

Blood of	Group and subgroup	M-N type	Rh-Hr type	
			Phenotype	Genotype
Father	O	M	Rh <sub>1</sub> Rh <sub>2</sub>	R <sup>1</sup> R <sup>2</sup> or r <sup>1</sup> R <sup>1</sup>
Mother	A <sub>1</sub>	M	rh	rr
Son	A <sub>1</sub>	M	Rh <sub>2</sub>	R <sup>2</sup>

TABLE 14

Week of pregnancy	Titer by agglutination technic	Titer by albumin-plasma conglutination technic
6th week	0	15
13th week	0	32
22nd week	0	20
17th week	0	40
30th week	0	23
34th week	0	112

Test for Rh antibodies on the mother's serum gave the following results. Agglutination test—negative, Blocking test—positive 2 units. Plasma conglutination test—positive 30 units. These results confirmed the diagnosis of erythroblastosis as the cause of the death of the third infant, and as the cause of the stillbirth which occurred in the fourth pregnancy. In view of the presence of a high titer of univalent antibodies and the fact that the husband belonged to type Rh<sub>1</sub>Rh<sub>2</sub>, every child of this couple would almost surely be Rh positive and stillborn, so the parents were advised against further pregnancies.

The mother was seen again in the sixth week of her fifth pregnancy and from that point on repeated titrations were done until delivery. The results of these studies are shown in table 14. At the time that the last antibody test was done examination by the attending obstetrician revealed that hydramnios had developed.

*Prognosis.* In view of the high titer of univalent antibodies and the development of hydramnios indicating fetal pathology, there was little hope of saving this baby unless delivery was carried out immediately and exchange transfusion performed. Intrauterine death seemed to be imminent if the pregnancy were to be allowed to continue for even a few more days.

*Procedure.* Delivery by cesarean section was carried out at thirty six weeks. The infant was pale and

icteric at birth and weighed 5 pounds 8 ounces. Blood was taken for tests to be carried out subsequently and immediate exchange transfusion was performed, using fresh citrated blood from an A, rh donor. One half of the plasma was removed from the donor's blood and replaced by an equal volume of normal saline. Over a period of 90 minutes 440 cc. of blood were injected and 380 cc. removed. The baby withstood the procedure well but was having respiratory difficulty when it was sent to the nursery.

**Results.** The hemoglobin concentration was 9.0 grams per cent at birth with a red blood cell count of 2.2 million per cu. mm. The infant belonged to group O type M type Rh<sub>1</sub>rh. The coagulation test for coating of the infant's erythrocytes were positive and free univalent antibodies could also be detected in the baby's serum.

Following the transfusion the hemoglobin concentration of the blood was 9.4 grams with a red blood cell count of 3.9 million per cu. mm. There were 164 nucleated red blood cells per 100 white blood cells on the smear. For two days the jaundice deepened gradually and the baby became lethargic and began to take its feedings poorly. On the third day of life the hemoglobin concentration had risen to 10.6 grams per cent with a red blood cell count of 4.7 million and there was still 200 nucleated red blood cells per 100 white cells on the smear. The jaundice deepened, the infant began to ooze blood from the mouth, developed respiratory distress and expired.

Through mischance this baby was left with a hemoglobin concentration of only 9.4 grams per cent at the end of the procedure, and this, we believe, contributed to its death. While in other cases following the exchange transfusion the erythroblasts quickly disappeared from the baby's blood stream, in this case they increased in number, possibly due in part to the anemia. A sample of blood obtained post-mortem showed twice as many Rh-positive cells as the immediate post-transfusion sample, while in our successful cases differential agglutination tests show no Rh-positive blood cells on the third day. The increase in the proportion of the Rh-positive cells nullified the procedure, and prevented recovery of the baby.

**CASE 13.** The mother of this patient was first seen by us in the twenty-fourth week of her second pregnancy because she had been found to be Rh negative in routine prenatal tests done elsewhere. Her first pregnancy had terminated spontaneously at term four years before with the birth of a male infant who is now well. This child had no anemia or jaundice. There was no history of the mother having had a transfusion or injection of blood or plasma.

**Findings.** Grouping and Rh Hr tests done on the father, mother and son gave the results shown in table 15.

Tests for antibodies were done on the mother's serum at intervals during the remainder of her pregnancy with the results shown in table 16.

**Prognosis.** These results indicated that the mother had become strongly sensitized to the Rh factor with antibodies predominantly of the bivalent type, and that the infant would probably have severe erythroblastosis.

**Procedure.** Plans were made to deliver the baby prematurely and perform an immediate exchange transfusion. However, the mother went into labor spontaneously in the thirty-fifth week of pregnancy and delivered a female infant who weighed 5 pounds, 15 ounces, was pale, but not icteric and could be resuscitated only with great difficulty. Immediate exchange transfusion was carried out with the administration of 560 cc. of blood from a donor who belonged to group O type rh and the removal of 460 cc. of blood. The baby withstood the procedure well.

**Results.** Examination of the infant's blood taken immediately after birth showed the hemoglobin concentration had been only 4.3 grams per cent. The albumin plasma coagulation test for coating of the infant's cells was positive and the serum of the cord blood showed the presence of free Rh antibodies in a titer of 3 units by the albumin plasma technique. On the day following the transfusion the hemoglobin concentration of the blood was 13.5 grams, the red blood cell count 4.2 million per cu. mm. and the white cell count 20,650. There were 220 nucleated red blood cells on the smear. The infant on that day began to show slight jaundice, there was some edema of the extremities and respirations were grunting and rapid. Five râles were audible throughout the entire chest, and cyanosis developed when oxygen

therapy was discontinued for feedings. Forty-eight hours after the procedure the picture became alarming. The liver and the spleen were both firm and readily palpable about three centimeters below the costal margins, petechiae were present over the shoulders and extremities and marked jaundice had developed. The hemoglobin concentration was now 13 grams per cent and there were 250 nucleated red blood cells per 100 white blood cells on the smear. The infant was given a transfusion of 60 cc. of group O, type rh blood and seemed to show some improvement for several hours. On the third day of life, however, dyspnea became more severe, the jaundice appeared to be deeper and the child refused all of its feedings. Blood taken several hours before death showed a serum bilirubin of 12.2 mg. per cent, and a prothrombin time of over three minutes as compared with the control of twelve seconds.

The findings at autopsy were kernicterus, cholemic nephrosis and pulmonary congestion, edema and atelectasis with foci of hematopoiesis in the liver and spleen.

CASE 14 This case is reported in detail elsewhere.<sup>10</sup>

CASE 15—This infant was first seen by us a few hours after birth because of severe anemia. Delivery had been spontaneous and at term. Pallor was noted immediately, and the amniotic fluid was seen to be

TABLE 15

Blood of	Group	M N type	Rh-Hr type	
			Phenotype	Genotype
Father	O	MN	Rh <sub>1</sub> Rh <sub>1</sub>	R <sup>1</sup> R <sup>1</sup> or R <sup>1</sup> r
Mother	O	MN	rh	rr
Son	O	MN	Rh <sub>1</sub> rh	R <sup>1</sup> r

TABLE 16

Time of test	Titer by agglutination technique	Titer by plasma-conglutination technique	Titer by albumin plasma technique
24 weeks	0	0	0
30 weeks	30	27	—
34 weeks	42	64	20

yellow. The infant's hemoglobin at birth was 5.8 grams per cent with a red blood cell count of 1.25 million. This was the mother's first pregnancy. She had no history of having had any abortions or miscarriages. Ten years before she had had poliomyelitis and was given a blood transfusion at that time.

Findings: Grouping and Rh-Hr tests as subsequently determined on the mother, father and the newborn infant gave results shown in table 17.

Tests done on the mother's serum for the presence of antibodies showed that the blocking test was positive in a titer of 4 units while the albumin plasma conglutination technique gave a titer of 100 units. Coombs' antiglobulin test for coating of the baby's erythrocytes was positive and the infant's plasma contained free polyvalent antibodies in a titer of 40 units by the albumin-plasma conglutination technique. The icterus index of the baby's serum was 40 units.

Prognosis: Although the above information was not available to us at the time that the baby was first seen, it was clear that the child was severely affected with erythroblastosis and the prognosis was very poor.

Procedure: Exchange transfusion was performed about five hours after birth. Because of the need for haste, group O type rh bank blood was used. One third of the plasma was removed and replaced with an equal quantity of normal saline. The infant received 500 cc. of blood while 450 cc. were removed.

Results: There was an immediate improvement in the baby's condition following transfusion but on

the following day, the patient again looked pale and was given another 75 cc of blood from a donor who belonged to group O, type rh. On the third day following the exchange transfusion jaundice developed and deepened rapidly. The hemoglobin concentration had now fallen to 10.1 grams per cent from the level of 12.3 grams that was present immediately following the exchange transfusion, and a second supplementary transfusion of 75 cc of fresh blood was given. Progress was not satisfactory, however. The icterus deepened, the liver and spleen became enlarged and the baby became lethargic and had intermittent periods of cyanosis. At the end of one week the temperature rose to 103 F and the anterior fontanelle was found to be bulging. Spinal tap revealed a canary yellow fluid that contained 7 white blood cells and 5 red blood cells per cu mm. The Pandy reaction was 4 plus and the qualitative sugar reaction on the spinal fluid was 3 plus. The blood culture was positive for staphylococcus aureus. Despite penicillin therapy, a large abscess which yielded 8 cc of purulent fluid on incision and drainage appeared over the upper thoracic vertebrae. Culture of this material was also positive for staphylococcus aureus. The baby developed diarrhea and scattered indurated areas over the body on the twenty ninth day of life and expired the following day.

Autopsy revealed bacteremia, pyohydrocephalus, abscesses in the thyroid, heart, kidney and the brain, kernicterus cirrhosis of the liver, septal thrombophlebitis of the pulmonary veins, and focal pneumonia.

TABLE 17

Blood of	Group	M N type	Rh-Hr type	
			Phenotype	Genotype
Father	A <sub>1</sub>	MN	Rb <sub>1</sub> Rb <sub>1</sub>	R <sup>1</sup> R <sup>1</sup> or R <sup>1</sup> r
Mother	A <sub>1</sub>	N	rh	rr
Baby	O	MN	Rh <sub>1</sub> rb	R <sup>1</sup> r

### Cases of Moderate Severity

CASE 16—This case has been reported in detail elsewhere.<sup>5</sup>

CASE 17—The mother of this patient was first seen by us in the first trimester of her third pregnancy. She had delivered a normal boy seven years previously who is living and well. Her second child, a boy, was born three years ago. He was normal at birth, but on the second day of life was seen to be jaundiced. The hemoglobin concentration of his blood was 7.3 grams per 100 cc and the red count was 2.3 million per cu mm. He was found to be Rh positive while the mother was Rh negative. Over a period of twelve days he received three transfusions of 75 cc each of group A, Rh negative blood without showing any appreciable rise in hemoglobin concentration or red blood count. On the fifteenth day of life, however, he was given 105 cc of washed mother's red cells in two portions and his hemoglobin concentration rose to 12 grams per cent. From this point onward his recovery was uneventful. At the time when his jaundice was at its peak the van den Bergh reaction showed a concentration of 46.7 mg of bilirubin in his blood.

*Findings.* Grouping and Rh-Hr tests done on the family gave the results in table 18.

At the time of the first test for Rh antibodies in the mother's serum no agglutinins were demonstrable, but univalent antibodies were shown to be present in a titer of 16 units by the plasma coagulation technique. Anti A and anti B titrations on the mother's plasma by both the agglutination and coagulation techniques were within normal limits. By the middle of the third trimester of pregnancy, however, Rh agglutinins could be demonstrated to a titer of 1½ units, while the coagulation titer had fallen to 3 units. A slight rise of both anti A and anti B titers above the normal was found at this time.

*Prognosis.* Since the anti Rh titer had fallen it could be confidently predicted that a viable infant would be obtained, even though the infant was almost certain to be Rh positive and therefore erythroblastotic. If the baby belonged to group A, the presence of mild sensitization to the A agglutino-gen might further complicate the picture, but probably not to a serious degree.

*Procedure.* The baby was delivered at term and was observed for twelve hours. Studies done during this time showed that the hemoglobin concentration of the blood was 14.5 grams per 100 cc. The red blood



count was 4.7 million cells per cu. mm. and the white blood count 26,200 per cu. mm. There were 20 nucleated red blood cells per 100 white blood cells and the icterus index of the cord serum was 12 units. The infant proved to belong to group  $A_2MNRh_1rh$ . After twelve hours the icterus index had risen to 24 units and the baby began to show slight clinical jaundice.

Exchange transfusion was carried out using a donor that belonged to group  $A_1MNRh$ . To reduce the concentration of the conglutinin in the infusion material, half of the plasma was removed from this blood and replaced with saline. Ten cc. of Wittebsky group substances were then added to neutralize the alpha antibodies present in the infant's body and derived from the mother. The usual procedure was then carried out, 500 cc. of blood being injected and 460 cc. simultaneously removed.

**Results.** The hemoglobin concentration was 13.5 grams per 100 cc. on the day following the transfusion. By the sixth day it fell to 8.8 grams per cent and another transfusion of 70 cc. of blood this time from an  $A_2rh$  donor was given. The hemoglobin rose to 12.7 grams per cent but over the next five days fell again to 8.7 grams. Following a final transfusion of 60 cc. of  $A_2rh$  blood the hemoglobin concentration became stabilized and the baby was discharged from the hospital. The van den Bergh reaction, which was indirect, showed a concentration of 5.7 mg. of bilirubin per 100 cc. at 3 days of age and fell steadily to normal during the hospital stay of three weeks. At the age of 2 months the hemoglobin concentration was 10.7 grams per cent and the baby was well. Some splenic enlargement was noted at that time but

TABLE 18

Blood of	Group and subgroup	M N type	Rh Hr type	
			Phenotype	Genotype
Father	A	MN	Rh <sub>1</sub> Rh	$R^1R^2$ or $r^1R^1$
Mother	O	N	rh	$r^1r$
1st son	O	MN	Rh <sub>2</sub>	$R^2r$
2nd son	A	N	Rh	$R^2r$

was no longer demonstrable 6 months later. At the age of one year the child was perfectly normal in every respect. He stood and was beginning to take a few steps. Language development was normal for that age.

This case is unusual in that the patient required two supplementary simple transfusions after the exchange transfusion, while in typical cases the exchange transfusion alone is sufficient to bring about a cure. This may be ascribed to the fact that the mother was sensitized to the agglutinin A as well as Rh, and especially to the use of blood of subgroup  $A_1$  for the exchange transfusion instead of blood of subgroup  $A_2$  or group O. The titer of alpha antibodies in the maternal serum was only slightly elevated, so that ordinarily one would expect the alpha antibodies passing into the fetal circulation to be completely neutralized by the A substance in its blood and tissues, leaving no free alpha antibody to affect the transfused group A blood cells. In this case, however, the baby belonged to subgroup  $A_2$ , so its tissues and blood were capable of neutralizing only the common alpha antibody, leaving alpha<sub>1</sub> antibody free to lyse the transfused  $A_1$  cells. While this prolonged the baby's illness, recovery readily resulted after two simple, supplementary transfusions of blood of subgroup  $A_2$ .

**CASE 18.**—No antenatal tests had been done in this case. The infant was referred to us when she was 9 hours old because of jaundice and anemia. She was the product of the second pregnancy. The first pregnancy was uncomplicated and resulted in the birth of a normal child who is living and well today. The

patient was born at term and was definitely icteric at birth. At the age of 5 hours the hemoglobin concentration was only 8.1 grams per cent and there were 15 normoblasts per high power field on the blood smear. The baby was given 80 cc of group O type rh blood before she was referred to us.

*Findings.* Grouping and Rh Hr tests on the family gave the results shown in table 19.

Antibody tests done on the mother's serum showed that a mixture of both bivalent and univalent antibodies were present. The agglutination test showed a titer of Rh antibodies of 12 units while the titer by the albumin plasma conglutination technic was 28 units. The serum of the infant was shown to contain free univalent Rh antibodies by the albumin plasma conglutination test which was positive to a dilution of 1:2.

*Prognosis.* In view of the significant antibody titer and deepening jaundice (icterus index 22.0 units) this was a severely affected infant who required immediate and vigorous treatment.

*Procedure.* Exchange transfusion was carried out twenty-four hours after birth using bank blood from an O, rh donor from which half of the plasma had been removed and replaced by saline. The baby was given 560 cc of this blood while 510 cc were removed.

*Results.* On the day following the transfusion the baby seemed very much improved although the hemoglobin concentration of the blood was only 11.7 grams per cent. At eighteen hours after the procedure the baby again became deeply jaundiced and edema of the extremities, particularly the legs developed. The spleen and liver were now enlarged and examination of the blood smear revealed that there were 270 nucleated blood cells per 100 white blood cells. At the same time the hemoglobin concen-

TABLE 19

Blood of	Group and subgroup	M V type	Rh Hr type	
			Phenotype	Genotype
Father	A <sub>1</sub>	MN	Rb <sub>1</sub> Rb <sub>1</sub>	R <sup>1</sup> R <sup>1</sup> or R <sup>1</sup> r'
Mother	A <sub>1</sub>	N	rh	rr
1st child	A <sub>1</sub>	MN	Rb <sub>1</sub> rh	R <sup>1</sup> r
Patient	O	—	Rb <sub>1</sub> rh	R <sup>1</sup> r

tration had fallen to 9.0 grams per cent. This evidence of continued blood destruction was interpreted as being due to the fact that bank blood had been used for the exchange and a further small transfusion of 40 cc of fresh blood from an O, rh donor was given. The jaundice began to fade on the following day and by the next week had faded completely. The hemoglobin concentration which had risen to 12.7 grams per 100 cc following the supplementary transfusion was maintained and the baby was discharged from the hospital at the age of two weeks. No further transfusions were necessary. When the baby was last seen at the age of 8 months she was sitting alone and appeared to be normal in every respect.

This is one of the few cases in which a supplementary simple transfusion was necessary after the exchange transfusions were done. This we ascribe to the use of bank blood of uncertain state of preservation. We were compelled to use bank blood in this case because no compatible donor was available, and the baby's critical condition made it imperative to avoid any delay in starting the exchange transfusion. The case demonstrates that for exchange transfusion fresh citrated blood, if available, should be used instead of bank blood because the more satisfactory results with fresh blood more than compensate for its greater cost and inconvenience.

*CASE 19.*—The mother of this infant was first seen by us in the thirty-first week of her fifth pregnancy. Her first pregnancy four years previously resulted in the birth of a son who is living and well. Her second pregnancy terminated with the birth of a girl who is also normal. Her third baby, a girl, was well until

the fourth day of life when she became jaundiced and anemic. She received four transfusions of Rh negative blood over a period of two weeks and made a complete recovery. The birth of this baby was followed by a miscarriage at two months. There was no history of the mother ever having received a blood or plasma transfusion or blood injection.

*Findings* Grouping and Rh Hr tests done on the father, mother and all the living children gave the results shown in table 20.

Tests for Rh antibodies on the mother's serum showed that while the agglutination test was negative, the titer of univalent antibodies was 4 units as determined by the albumin plasma conglutination test.

*Prognosis* Since the father was almost surely homozygous for the Rh<sub>0</sub> factor the new baby would be expected to be Rh positive and therefore erythroblastotic, though not severely affected in view of the rather low Rh antibody titer of the maternal serum.

*Procedure* It was planned to deliver the infant at term and do an exchange transfusion immediately after birth. However, the mother went into labor spontaneously six weeks before term and delivered a 5 pound premature infant that seemed to be normal. Exchange transfusion was performed using 380 cc. of group O, type rh bank blood for the infusion and removing 300 cc. of blood from the baby. In this particular case the blood vessels were found to be uncommonly small and difficulty was encountered with the bleeding so that we actually were obliged to fall short of the 500 cc. mark that we had established for ourselves as the minimal goal in doing an exchange transfusion.

TABLE 20

Blood of	Group	M V type	Rh Hr type	
			Phenotype	Genotype
Father	O	MN	Rh <sub>1</sub> Rh <sub>2</sub>	R <sup>1</sup> R <sup>2</sup> or R <sup>4</sup> *
Mother	O	N	rh	r
Son	O	N	Rh <sub>1</sub> rh	R <sup>1</sup> r
1st daughter	O	MN	Rh <sub>1</sub> rh	R <sup>1</sup> r
2nd daughter (erythroblastotic)	O	MN	Rh <sub>1</sub> rh	R <sup>1</sup> r

*Results* The baby did well from the clinical point of view, that is feedings were taken normally and the weight gain was satisfactory. The baby did not develop any jaundice. However the hemoglobin concentration which was 16 grams per cent at birth was only 13.4 grams per cent on the day following the transfusion. Over the next eleven days the hemoglobin concentration fell to 10.5 grams per cent and the infant was discharged. Four days later the hemoglobin concentration of the blood had fallen to 8.0 grams and the patient was transfused with 55 cc. of group O, type rh bank blood. Two days later the transfusion was repeated and the hemoglobin concentration from that point onward was maintained at over 11 grams per cent. The child's subsequent course has been uneventful, though there is some slight doubt in the mind of the mother that he is as bright as his siblings.

*Case 20*—This one day old female infant was referred to us because of jaundice and anemia of six hours duration. She was the second child. The first a girl was 3½ years of age and was well. The mother had never been transfused and had never had any stillbirths or miscarriages.

*Findings* When first seen the infant was deeply jaundiced and had numerous petechiae on the forehead. The liver and spleen were not enlarged. The hemoglobin concentration of the blood was 12.3 grams per cent and red blood count was 3.4 million per cu. mm. The icterus index was 112 units.

Grouping and Rh Hr tests done on the mother, father and the patient gave the results shown in table 21.

Antibody tests done on the mother's serum were positive to 1½ units by the agglutination technic, and also by the plasma conglutination and albumin plasma techniques. Conglutination tests for coating of the baby's erythrocytes were negative and no free Rh antibody was demonstrable in the infant's serum.

*Progress* If one could depend entirely upon the maternal antibody titer as a criterion, this could be regarded as a case with only mild sensitization and therefore with a good prognosis. However the severe clinical condition of the infant, with deep jaundice and hemorrhagic phenomena called for more vigorous treatment than simple transfusion. We felt that with exchange transfusion the prognosis would be good and also that the need for repeated transfusions would be obviated.

*Procedure* Group O type rh bank blood was used. One half of the plasma was removed and replaced with normal saline. The baby received 450 cc while 450 cc was removed over a period of about ninety minutes. The baby withstood the procedure well.

*Results* On the following day the hemoglobin concentration of the blood was 13 grams per cent and the red blood cell count was 4.25 million per cu mm. There were 3 normoblasts per 100 white blood cells on the smear. The jaundice faded in three days and the child was discharged as well.

*CASE 21*—The mother of this patient was first seen in the thirty-sixth week of her third pregnancy. She had had a spontaneous miscarriage at 4½ months two years previously, and a spontaneous abortion at

TABLE 21

Blood of	Group and subgroup	M N type	Rh Hr type	
			Phenotype	Genotype
Father	O	N	Rh <sub>1</sub> rh	R <sup>1</sup> r or R <sup>1</sup> R <sup>0</sup>
Mother	B	MN	rh	rr
Patient	O	N	Rh <sub>1</sub> rh	R <sup>1</sup> r

TABLE 22

Blood of	Group and subgroup	M N type	Rh Hr type	
			Phenotype	Genotype
Expectant father	A <sub>1</sub>	MN	Rh <sub>1</sub> Rh <sub>1</sub>	R <sup>1</sup> R <sup>1</sup> or R <sup>1</sup> r'
Expectant mother	O	MN	rh	rr

2½ months about one year later. Three years ago she was operated upon for volvulus of the small bowel and had had 8 feet of ileum removed. She was given a blood transfusion postoperatively.

*Findings* Grouping and Rh Hr tests done on the prospective parents gave the results shown in table 22.

Test for Rh antibodies done on the mother's serum gave the results shown in table 23.

In view of the difference in the blood groups, titrations were also carried out for alpha and beta antibodies in the mother's serum. The results of these tests are shown in table 24.

*Prognosis* From the results of the Rh antibody tests, it was evident that the mother was definitely though weakly, sensitized to the Rh factor, and that it was likely that the expected infant would have mild erythroblastosis.

*Procedure.* In order to spare the infant prolonged contact with the antibodies it was planned to induce labor about two weeks before term and treat the infant with exchange transfusion but only if signs of erythroblastosis developed. The obstetrician elected to deliver the patient by cesarean section and this was carried out at the end of the thirty-eighth week of pregnancy.

The infant, a male, weighed 6 pounds and 1 ounce and appeared to be normal. There was no pallor, jaundice or hepatosplenomegaly. The hemoglobin concentration of the blood was 16.9 grams per cent and the red blood cell count was 4.14 million per cu mm. There was 8 normoblasts per white blood cells on the smear. The icterus index was 14 units and the baby's blood typed as AMNRh<sub>1</sub>rh.

Twenty-four hours after birth, slight jaundice was noted and blood studies showed that the hemoglobin concentration had now fallen to 13.5 grams per cent with a red blood cell count of 3.4 million per

cum mm Five normoblasts per 100 white blood cells were seen on the smear. No hepatic or splenic enlargement had developed. The serum bilirubin concentration was 9.5 mg/100 cc.

Exchange transfusion was performed with the administration of 520 cc. blood from an A<sub>3</sub>Nth donor, and the removal of 470 cc. The baby tolerated the procedure well.

**Results**—On the day following the procedure the hemoglobin concentration of the blood was 13 grams per cent and the red blood cell count 4.28 million per cu. mm. The jaundice was unchanged and the infant appeared well.

At this time there was an outbreak of diarrhea on the ward, and despite all precautions the patient developed loose watery stools and rapidly became dehydrated and acidotic. The CO<sub>2</sub> content of the blood fell to 22 volumes per cent and the patient was treated with starvation and parenteral fluids. Blood culture was negative and the stool culture was negative for pathogens. Feedings were resumed after twenty-four hours when the character of the stools returned to normal. Two days after the onset of diarrhea the hemoglobin concentration of the blood had fallen to 11 grams per cent and the red blood cell count to 3.9 million per cu. mm. The patient was transfused twice with 60 cc. blood from group O type Rh donors, and after the diarrhea was completely controlled was discharged 17 days after admission.

When seen at the age of 2 months he was well. He weighed 10 pounds and was not jaundiced.

TABLE 23

Time of test	Titer by agglutination technic	Titer by albumin plasma technic
36th week	0	doubtful
37th week	0	1½

TABLE 24

Time of test	Agglutination technic		Plasma-conglutination technic	
	Anti A	Anti B	Anti A	Anti B
36th week	96	60	64	80
37th week	40	48	80	48

### Mild Cases

**CASE 22**—The mother of this infant was first seen in the thirty-fifth week of her third pregnancy. Her first pregnancy had terminated prematurely with the birth of a normal female child. Her second infant, a female, was carried to term and was delivered normally. Both of these children are living and well. There was no history of the mother ever having received a transfusion or blood injection.

**Findings**—Grouping and Rh Hr tests done on the father, mother, and both children gave the results shown in table 25.

The results of the antibody titrations done on the mother's serum are given in table 26.

**Prognosis**—These findings indicated that the expected infant would almost surely be Rh positive and therefore erythroblastic, since the mother was sensitized to the Rh factor. However, since the Rh agglutinins in the maternal serum interfered with the determination of the titer of univalent antibodies, if any, the severity of the manifestations in the baby were not predictable.

**Procedure**—Labor was induced in the thirty-ninth week of pregnancy and the infant, a female, was immediately treated by exchange transfusion. Over a period of one hour 500 cc. of blood from an O Rh donor were injected and 480 cc. removed. The baby stood the procedure well.

**Results**—No clinical symptoms of erythroblastosis ever developed. The baby's hemoglobin concentration at birth was 17.4 grams per cent and the red blood cell count 4.3 million. There were no erythroblasts on the smear. Coating test on the baby's red blood cells (OMNRh<sub>2</sub>) was negative. On the day following

the transfusion the hemoglobin concentration was 15.5 grams. Mild icterus made its appearance on the second day of life but faded rapidly thereafter. The baby was discharged on the fourth day.

**CASE 23**—This was the mother's second pregnancy. Her first pregnancy was normal and went to term but labor was prolonged and the infant was delivered by high forceps. The child had no jaundice or anemia but its neonatal period was complicated by convulsions said to be due to cerebral hemorrhage attendant upon the traumatic delivery. Routine Rh tests done in the course of the second pregnancy revealed the mother to be Rh negative and sensitized to the Rh factor.

**Findings** Grouping and Rh Hr tests were done on the family, and the results are shown in table 27.

Antibody tests on the mother's serum done at thirty-six weeks were positive to a titer of 2 units by the agglutination technic and to a titer of 14 units by the albumin plasma conglutination technic.

TABLE 25

Blood of	Group and subgroup	M \ type	Rh Hr type	
			Phenotype	Genotype
Father	O	N	Rh Rh	$R^+R^+$ or $R^+r$
Mother	O	M	rh	rr
1st daughter	O	MN	Rh <sub>2</sub> rh	$R^+r$
2nd daughter	O	MN	Rh rh	$R^+r$

TABLE 26

Week of pregnancy	Titer by agglutination technic	Titer by albumin plasma technic
35 weeks	6	6
39 weeks	36	20

TABLE 27

Blood of	Group and subgroup	M \ type	Rh Hr type	
			Phenotype	Genotype
Father	A <sub>1</sub>	MN	Rh <sub>1</sub> Rh <sub>1</sub>	$R^+R^+$ or $R^+r$
Mother	B	M	rh	rr
First child	O	M	Rh <sub>1</sub> rh	$R^+r$

**Prognosis** Since the husband was almost surely homozygous for the Rh<sub>0</sub> factor every child of this couple was bound to be Rh positive. In view of the moderately high titer of univalent antibodies in the mother's circulation an erythroblastotic infant with manifestations of only moderate severity was to be expected.

**Procedure** In order that the infant be spared unnecessarily prolonged exposure to the Rh antibodies delivery was carried out about 10 days before term. This was done by cesarean section because of the mother's contracted pelvis. Sterilization by ligation of the fallopian tubes was also done at this time. At birth the infant appeared to be perfectly normal clinically. The baby was grouped and was found to belong to group AB type Rh<sub>1</sub>rh and so was Rh positive as expected. Exchange transfusion was performed using blood from a group AB type rh donor. Over a period of forty minutes 560 cc. of blood were injected while 520 cc. were removed. The baby withstood the procedure very well. Studies done on the cord blood showed a hemoglobin concentration of 18 grams per 100 cc. with a red blood count of 5.75 million. No nucleated red cells were seen on the smear. However, the conglutination test for coating of infant's red cells by antibodies was positive.

*Results* The baby never developed either jaundice or anemia. It was discharged from the hospital on the 10th day and required no further transfusions. Differential agglutination studies showed that a 90 per cent replacement had been effectuated. At 3 weeks postpartum the titer of anti Rh agglutinins in the mother's serum had risen to 88 units while the antibody titer was shown to be 175 units by the albumin-plasma conglutination technic.

In most sensitized Rh-negative women, there is a rise in Rh antibody titer following the birth of the baby, probably due to leakage of infant's blood into the maternal circulation during labor. The case just described demonstrates that such a rise also occurs when delivery is accomplished by cesarean section, so that operative delivery does not prevent maternal sensitization. In view of the very high Rh antibody titer of the maternal serum after delivery and the fact that the husband belonged to type Rh<sub>1</sub>Rh<sub>1</sub>, it seems obvious that every future pregnancy would almost surely result in death of the fetus before it reached the stage of viability.

*CASE 14*—This infant was referred to us at the age of 2 days because of jaundice which had first been noticed when the baby was 17 hours old. The mother had had two previous pregnancies, and both children were alive and well. The first had been entirely normal during its neonatal period while the

TABLE 18

Blood of	Group and subgroup	M N type	Rh Hr type	
			Phenotype	Genotype
Father	A <sub>1</sub>	N	Rh <sub>1</sub> Rh <sub>1</sub>	R <sup>1</sup> R <sup>1</sup> or R <sup>1</sup> r'
Mother	O	N	rh	rr
1st son	O	N	Rh <sub>1</sub> rh	R <sup>1</sup> r
2nd son	O	N	Rh <sub>1</sub> rh	R <sup>1</sup> r
Patient	A <sub>1</sub>	N	Rh <sub>1</sub> rh	R <sup>1</sup> r

second had developed jaundice on the second day of life and recovered after a brief illness. There was no history of the mother's ever having received any blood transfusions. Antenatal Rh testing had not been done.

The patient appeared to be normal at birth but jaundice was noted on the morning of the second day of life. The hemoglobin concentration was found to be 10.2 grams per cent and the icterus index 50 units.

*Findings* Forty-eight hours postpartum grouping and Rh Hr tests done on the family gave the results shown in table 18.

Antibody studies done on the mother's plasma showed that while no Rh antibodies could be demonstrated by the saline agglutination technic, weak univalent antibodies were present in a titer of 3 units as demonstrated by the albumin plasma method. Furthermore, univalent antibodies could be demonstrated in the infant's serum also in a titer of 2 units. Since there was a possibility of double sensitization (to A as well as to Rh) the mother's anti A and anti B titers were determined. By the agglutination method anti A was demonstrable in her serum in a titer of 40 units, and anti B in a titer of 12 units. By the plasma conglutination technic the titer of anti A was 60 units and anti B was 30 units.

*Prognosis* This then was a case of double though mild, sensitization to both the Rh factor and the A agglutino-gen. If untreated, the infant was bound to develop a mild but progressive anemia that would require several transfusions if treated in the usual manner. In view of the low antibody titers there was practically no danger of intravascular clumping.

*Procedure* In order to limit the number of transfusions required, an exchange transfusion was decided upon. Four hundred cc. of blood were withdrawn from the infant and simultaneously replaced by 500 cc. of blood from a group A type rh donor. Following the transfusion 10 cc. of Witte's A and B group





Half of the plasma was removed and replaced with saline. Five hundred and forty cc. were injected and 480 cc. withdrawn. The baby withstood the procedure well.

*Results:* At the end of the transfusion the icterus index was 45 units and on the day following had fallen to 30 units. The jaundice subsided rapidly and the baby was discharged from the hospital at the end of one week. At the age of 3 weeks the hemoglobin concentration of the baby's blood was 14.4 grams per cent. At the age of 3 months the child was doing well and seemed to be developing normally.

**CASE 26**—The mother of this infant was first seen by us in the thirty-seventh week of her second pregnancy. Her first pregnancy had terminated with the birth of a male infant who is well. Following the delivery the mother had a pulmonary embolus from which she did not recover for several months. She never had a blood or plasma transfusion. She had been found to be Rh negative by routine antenatal Rh testing but no antibodies had been found until a few days before she was referred to us.

*Findings:* Grouping and Rh Hr tests done on the father, mother, and son gave the results shown in table 31.

No agglutinins could be detected in the mother's serum and univalent antibodies of only one unit titer were found to be present by the albumin plasma technic.

*Prognosis:* Since the father was almost surely homozygous for the Rh<sub>0</sub> factor, the expected infant would be Rh positive. However, the low titer of antibodies in the mother's serum made it questionable that the infant would be affected by the disease. In fact, if erythroblastosis did develop at all, the manifestations would be expected to be very mild.

*Procedure:* Labor was induced at the end of the thirty-eighth week of pregnancy and a normal appearing baby girl weighing 6 pounds was delivered. The icterus index of the cord blood was 14 units and the hemoglobin concentration of the blood was 12.9 grams per cent with a red blood cell count of 4.33 million per cu. mm. There was one normoblast per 100 white blood cells on the smear. No jaundice or hepatosplenomegaly was noted. The albumin plasma conglutination test for coating of the infant's cells was negative though as predicted the baby was Rh positive (OMNRh<sub>1</sub>rh).

Twelve hours later the icterus index had risen to 28 units and it was decided that an exchange transfusion be performed. Blood was drawn from a donor who belonged to group O type rh and one half of the plasma removed and replaced with normal saline to reduce the conglutinin content. Over a period of one and one half hours 500 cc. of blood were injected and 450 cc. removed.

*Results:* Twenty-four hours after the transfusion the hemoglobin concentration of the blood was 16.1 grams per cent and the red blood cell count 5.4 million per cu. mm. The baby did not develop jaundice or anemia while in the hospital and was discharged with the mother on the fourth day. At the age of one week the hemoglobin concentration was 13.8 grams per cent and the red blood cell count 4.66 million. There was no jaundice present clinically and by differential agglutination the baby's blood typed as 100 per cent type rh. One week after delivery the titer of univalent antibodies in the mother's serum had risen to 10 units by the albumin plasma technic. No agglutinins were demonstrated. At the age of one month the hemoglobin concentration of the infant's blood had fallen to 8.4 grams and then rose spontaneously to 9.6 grams at the age of 2 months and to 12.9 grams per cent by the age of 3 months. Blood typing at this time showed that all the erythrocytes typed as OMNRh<sub>1</sub>rh, the baby's original type. The infant's subsequent course has been uneventful.

In retrospect, we consider this as a case that would have done well with the usual transfusion therapy or might even have recovered without any therapy at all. This case occurred early in our series and we were unduly impressed with the slight anemia and the rise in icterus index that occurred after delivery. Subsequently, we have seen 4 cases which had similar minimal titers of antibodies in the maternal serum. Of these, 2 developed mild clinical signs of erythroblastosis and recovered without therapy. The other 2 had no clinical signs of the disease at all.

**CASE 27**—The mother of this patient was first seen by us six months after her first pregnancy. This had terminated one month prematurely with a stillborn anencephalic male and was complicated by placenta previa. Following the delivery she received four transfusions of blood. She had chills and high fever.

following the first two of these but no reactions to the third or fourth. It is not known whether the blood she received was selected on the basis of Rh testing.

*Findings* Grouping and Rh Hr tests done on the woman and her husband gave the results shown in table 32.

Tests for antibodies done on the wife's serum showed that while no antibodies could be demonstrated by the saline agglutination technique, univalent antibodies of 4 units titer were shown to be present by the plasma agglutination method.

Six months later the antibody studies were repeated and this time no antibodies could be demonstrated by either the agglutination or the plasma agglutination techniques.

About six months after these tests were done she became pregnant again and the results of antibody tests done on her serum throughout her pregnancy are shown in table 33.

TABLE 31

Blood of	Group and subgroup	M N type	Rh Hr type	
			Phenotype	Genotype
Father	O	M	Rh <sub>1</sub> Rh <sub>1</sub>	R <sup>1</sup> R <sup>1</sup> or R <sup>1</sup> r'
Mother	A <sub>1</sub>	MN	rh	rr
Son	O	M	Rh <sub>1</sub> rh	R <sup>1</sup> r

TABLE 32

Blood of	Group and subgroup	M N type	Rh Hr type	
			Phenotype	Genotype
Husband	A <sub>1</sub>	M	Rh <sub>1</sub> Rh <sub>1</sub>	R <sup>1</sup> R <sup>1</sup> or R <sup>1</sup> r'
Wife	A <sub>1</sub>	MN	rh	rr

TABLE 33

Week of test	Titer by agglutination technic	Titer by albumin plasma technic
9th week	0	0
22nd week	0	0
28th week	0	0
35th week	0	2

*Prognosis* Delivery was accomplished by cesarean section at the end of the thirty-seventh week of pregnancy. A blood count was done immediately after birth and showed that the hemoglobin concentration of the blood was 12.7 grams per cent and the red blood cell count was 3.8 million per cu. mm. There were 10 nucleated red blood cells per 100 white blood cells on the smear. In view of these findings an exchange transfusion was carried out with the administration of 500 cc. of group A type rh blood and the removal of 425 cc. of infant's blood.

*Results* The infant withstood the procedure well and on the following day the hemoglobin concentration was 17.1 grams per cent with a red blood cell count of 5.65 million. The baby never became jaundiced and was discharged on the eighth day. When seen at the age of 3 months the baby was well, had gained weight normally and appeared to be alert and active.

#### COMMENT

The case histories have been presented in considerable detail because it is only in relation to the severity of the individual case that the efficacy of the exchange

TABLE 34.—Summary of Cases Studied Antenatally

Case number	Father's Group and Rh Hr type	Mother's group and Rh Hr type	Maternal Rh antibody titer (units)		Infant's group and Rh Hr type	Clinical Summary see bottom of table Results
			Agglutination Method	Conglutination Method		
14	O Rh rh	A <sub>1</sub> rh	0	1400 (Bl 32)	O Rh <sub>2</sub> rh	Died on 2nd day
12	O Rh <sub>1</sub> Rh <sub>2</sub>	A <sub>1</sub> rh	0	112 (Bl 2)	O Rh <sub>1</sub> rh	Died on 3rd day
5	A <sub>2</sub> Rh <sub>1</sub> rh	O rh	0	44 (Bl 6)	A <sub>2</sub> Rh <sub>1</sub> rh	Recovered
7	O Rh <sub>1</sub> Rh <sub>1</sub>	A <sub>1</sub> rh	0	30	A <sub>1</sub> Rh <sub>1</sub> rh	Recovered
1	A <sub>1</sub> Rh <sub>1</sub> Rh <sub>2</sub>	A <sub>1</sub> rh	0	12*	A <sub>1</sub> Rh <sub>1</sub> rh	Recovered
19	O Rh <sub>1</sub> Rh <sub>2</sub>	O rh	0	4	O Rh <sub>1</sub> rh	Recovered
27	A <sub>1</sub> Rh <sub>1</sub> Rh <sub>1</sub>	A <sub>1</sub> rh	0	2	A <sub>1</sub> Rh <sub>1</sub> rh	Recovered
21	A <sub>1</sub> Rh <sub>1</sub> Rh <sub>1</sub>	O rh	0	1½	A <sub>1</sub> Rh <sub>1</sub> rh	Recovered
26	O Rh <sub>1</sub> Rh <sub>1</sub>	A <sub>1</sub> rh	0	1	O Rh <sub>1</sub> rh	Recovered
10b	O Rh <sub>1</sub> Rh <sub>1</sub>	O rh	32	26	O Rh <sub>1</sub> rh	Died edematous and deeply jaundiced after 24 hours
10a	O Rh <sub>1</sub> Rh <sub>1</sub>	O rh	12	25	O Rh <sub>1</sub> rh	Died at 6 days
13	O Rh <sub>1</sub> Rh <sub>1</sub>	O rh	42	20	O Rh <sub>1</sub> rh	Died at 3 days
22	O Rh <sub>2</sub> Rh <sub>2</sub>	O rh	36	20	O Rh <sub>2</sub>	Recovered
3	O Rh <sub>1</sub> Rh <sub>2</sub>	O rh	7	20*	O Rh <sub>2</sub>	Recovered
4	A <sub>1</sub> Rh <sub>1</sub> rh	A <sub>1</sub> B rh	16	14	B Rh <sub>1</sub> rh	Recovered
23	A <sub>1</sub> Rh <sub>1</sub> Rh <sub>1</sub>	B rh	2	14	AB Rh <sub>1</sub> rh	Recovered
9	A <sub>1</sub> Rh <sub>1</sub> rh	A <sub>1</sub> rh	6	10	A <sub>1</sub> Rh <sub>1</sub> rh	Recovered
25	A <sub>1</sub> Rh <sub>1</sub> Rh <sub>2</sub>	O rh	2	8	A <sub>1</sub> Rh <sub>1</sub> rh	Recovered
27	A <sub>2</sub> Rh <sub>1</sub> Rh	O rh	1½	3	A <sub>2</sub> Rh <sub>1</sub> rh	Recovered
11	—	—	64	—	—	Died a few hours after transfusion

\* All titrations by conglutination method in albumin plasma except cases indicated by asterisks which were done by plasma method

Abbreviations used II = icterus index Eb = erythroblastosis Bl = titer by blocking technic.

CLINICAL SUMMARY Case 14 Primipara Sensitized by pooled serum injections Cesarean section at 31 wks. Hb-6.3 Gm. cells coated free antibodies 400 units. Case 12. Previous stillbirth Cesarean section at 36 wks 5½ lbs Coated cells Hb-9 Gm. 164 nucl. RBC Case 5 Previous child kernicterus Normal term birth Cells coated Double sensitization Req supplement transf Case 7 Prev child died of Eb Labor induced at 38 wks Amn. fl. yellow Cells coated Free antibody in baby's serum. Case 1 Cesarean section 38 wks. Hb 11 Gm 69 nucl RBC. II 35 Cells coated Case 19 Previous child Eb Hb 16 Gm. Bank blood used. Slow fall in Hb to 10.5 in 11 days. 2 subsequent transf. Case 27 Sensitized by transfusion following birth of anencephalic monster Hb 12.7 Gm 10 nucl RBC No jaundice Case 21 Previous miscarriage and abortion Del by Cesarean section. Hb 16.9 Gm. II 14 Dev diarrhea and urinary tract infection. Case 26 Hb 12.9 II 14 No coagulation of cells Treated because of rise in icterus index. Case 10b Sibling of Case 10a Antibodies present thruout pregnancy Cesarean sect. 34 wks Cells coated 45 nucl. RBC Hb 5.8 Gms., II 52. Treated with 1,000 c.c. exchange Case 10a 2 previous stillbirths Cesarean section at 34 wks 5½ lbs Hb 11.7 18 nucl. RBC Cells coated II 70. Case 13 First child normal Spont deliv at 35 wks Hb 4.3 Gm 220 nucl RBC. Cells coated. Case 22. Hb 17.4 Gm No nucl RBC. No coating of cells. Negligible icterus Case 3 Five early miscarriages Sixth preg normal child. Hb 9.7 12 nucl RBC. II 40 Cyanosis and jaundice Case 4 Induced at 37 wks Hb 13.9 II 24. Severe jaundice for one week Case 23 Section for contracted pelvis at 39 wks Hb 18 Gm. No nucl RBC. Cells minimally coated. Case 9 Cesarean section at 37 wks Hb 17.4 Gm. II 281 nucl RBC. Cells coated Free antibody in baby's serum Jaundiced for one week Case 25 Induced at 37 wks Hb 18 Gm II. 10. Slight

transfusion can be evaluated. Unfortunately, no comparable series of cases subjected to other types of treatment, such as simple transfusion therapy, is available for comparison. As we and others have shown elsewhere,<sup>1, 13</sup> the most reliable indication of the severity of the disease is provided by antenatal studies of the Rh antibodies in the maternal serum as well as through studies of the Rh antibodies in the infant's blood. This is demonstrated in table 34 which summarizes those cases that were studied antenatally.

Before discussing table 34 a few words are necessary concerning the relative roles of the bivalent and univalent antibodies in the pathogenesis of erythroblastosis. Whereas originally our tendency was to ascribe almost equal importance to the two kinds of antibodies,<sup>5</sup> the demonstration that the intact placenta allows blocking antibodies (glutinins or univalent antibodies) to pass across freely while holding back agglutinins (bivalent antibodies) has convinced us that the latter play only a subsidiary role in the disease.<sup>7</sup> In fact, the presence of agglutinins in the maternal serum may be entirely misleading, and in one case seen by us recently with an agglutinin titer of more than 100 units, the Rh positive infant subsequently born showed hardly any evidence of erythroblastosis. On the other hand, we have encountered no case with a significant titer of univalent antibodies in which an entirely normal Rh-positive fetus was subsequently born. Nonetheless, the presence of agglutinins is of some significance since it indicates that the mother has been sensitized so that her serum may well contain univalent antibodies in addition. Unfortunately, agglutinins react equally well in plasma and saline media, so that unless the univalent antibodies contained in the same serum are of significantly higher titer their presence would not be demonstrable with the methods available at the time that our cases were studied.\* Based on this concept one would expect that the severity of the manifestations in the erythroblastic baby should depend upon the titer of the univalent antibodies in the maternal serum as well as upon the length of time that they were present antenatally. To demonstrate this, the cases have been arranged according to the titer of maternal univalent antibodies at the last test before delivery. For the reasons just discussed these cases are divided into two groups, depending upon whether or not antibodies were also demonstrated by the saline agglutination method.

As shown in table 34, the severity of the manifestations parallels the titer of univalent antibodies in the maternal serum. The 5 infants† who died comprise the 2 with the highest titers in the first group and the 3 with the highest titers in the second group. In case 22 in the second group, the infant was but mildly affected, though the titer of the maternal antibodies was relatively high, in this case, the maternal serum most likely contained agglutinins with only weak accompanying univalent antibodies.

In table 35 are summarized those cases in which antenatal tests had not been

\* Utilizing the differences in behavior of univalent and bivalent antibodies such as the difference in resistance to heat, simple methods have been devised whereby univalent antibodies can be detected despite the presence of strong agglutinins.<sup>24</sup>

† This does not include case 11, because we had no access to the mother of this baby and so could not do our own antibody studies.

coating of cells. Treated after rise in I.L. to 65 after 44 hours. Case 17: Previous infant hemolytic anemia. Pt. term birth. Double sensitization. Req. suppl. trans. with A rh blood. Case 11: Baby in desperate condition when seen at age of four days.

done, because the patients were not seen until the infants had developed obvious manifestations of erythroblastosis. Here again the correlation between the titer of maternal antibodies and the prognosis is apparent, since the only infant that died is the one whose mother had the highest antibody titer.

Observation of the infants treated by exchange transfusion immediately convinced us of the efficacy of the treatment so that we did not feel justified in withholding the treatment from any patient merely in order to set up a control series artificially. Since progress in the technics of demonstrating antibodies<sup>14</sup> has paralleled progress in therapy of the disease, even our own previous series do not constitute adequate controls because of incomplete serologic information. In

TABLE 35—*Cases Seen for the First Time after Birth*

Case Number	Father's Group and Rh-Hr type	Mother's Group and Rh-Hr type	Titer of Maternal Antibodies (units)		Baby's Group and Rh-Hr type	Clinical Summary see bottom of table Results
			Agglutination Method	Conglutination Method		
15	A <sub>1</sub> Rh <sub>1</sub> Rh <sub>1</sub>	A <sub>1</sub> rh	0	100 (Bl 4)	O Rh <sub>1</sub> rh	Persistent deep jaundice. Dev sepsis and died at 1 mo
6	O Rh <sub>1</sub> Rh <sub>1</sub>	A <sub>1</sub> rh	0	40 (Bl 1½)	O Rh <sub>1</sub> rh	Recovered
24	A <sub>1</sub> Rh <sub>1</sub> Rh <sub>1</sub>	O rh	0	3	A <sub>1</sub> Rh <sub>1</sub> rh	Recovered
2	B Rh <sub>1</sub> Rh <sub>1</sub>	O rh	20	70	O Rh <sub>1</sub> rh	Recovered
16	O Rh <sub>1</sub> rh	O rh	30	40	O Rh <sub>1</sub> rh	Recovered
8	A <sub>1</sub> Rh <sub>1</sub> rh	A <sub>1</sub> rh	8	40	O Rh <sub>0</sub>	Rapid recovery
18	A <sub>1</sub> Rh <sub>1</sub> Rh <sub>1</sub>	A <sub>1</sub> rh	12	28	O Rh <sub>1</sub> rh	Recovered
20	O Rh <sub>1</sub> rh	B rh	1½	1½	O Rh <sub>1</sub> rh	Very rapid recovery

CLINICAL SUMMARY *Case 15* Primip Transf 10 yrs prev Hb 5.8 Gm Cells coated LI 40, free antibody in baby's serum 40 units. *Case 6* Pallor Jaundice. (LI 60) Hb 11.4. Cells coated Free antibody Hepatosplenomegaly. *Case 24* Jaundice. Hb 10.2, LI 80 Cells coated Free antibody in baby's serum. *Case 2* Jaundice, shock, petechiae. LI 60 Hb 8.8 Gm Cells coated. *Case 16* Jaundice severe Hb 16.9 Gm. No erythroblasts. *Case 8* Normal at birth. Severe jaundice at 48 hrs Hb 11.7 Gms 7 nucl RBC. *Case 18* Jaundice, pallor, Hb 8.1 gms 15 nucl RBC., LI 220 Cells coated. Free antibody in baby's serum. *Case 20* Hb 12.3 gms, LI 112. Petechiae on forehead. Cells not coated

arranging the cases under the headings above, both the serologic findings and the severity of the clinical manifestations were taken into account, and it should be emphasized that in some of the severest cases (cases 2, 4, 7 and 8), the recovery after treatment was so rapid that it differed strikingly from anything experienced before this new type of treatment was instituted. It is true that 7 babies died despite treatment, but the clinical and serologic findings indicate that at least twice as many might have died if treated in the orthodox manner, namely, by multiple small transfusions of Rh-negative blood.

In the infants who responded to treatment the results were particularly gratifying for two reasons (1) The treatment besides being simple, was efficient, and only a few babies required supplementary treatment. In our opinion, it is simpler to do a single exchange transfusion than to do repeated simple transfusions, aside from the

greater efficacy of the former. In those cases that required supplementary transfusions, either bank blood had been used instead of fresh blood, or double sensitization (to A or B as well as Rh) was present. In some cases an intercurrent complication, unrelated to erythroblastosis, made further transfusions necessary. (2) All the infants who recovered developed normally, both physically and mentally, without any sequelae of liver or brain damage.

Some critics of the procedure have suggested that the infants who died succumbed to the effects of large amounts of citrate used as an anticoagulant in the transfused blood. Evidence<sup>19</sup> is available that the citrate is rapidly metabolized by the infants who survived and it is clear from the case histories that the infants that did not recover died in spite of, and not because of the treatment. The best disproof of the theory of citrate toxicity is our recent observations in which we were able to save infants by doubling the amount of blood used for the exchange transfusion despite the presence in the maternal serum of antibodies with titers which were uniformly lethal when only 500 cc. of blood were used. There is no doubt that there is some degree of toxicity when the exchange transfusion is done too rapidly and a temporary hypocalcemia results. This is readily counteracted by the cautious administration of calcium gluconate.\* If the infants show no hypocalcemic symptoms at the termination of the procedure no delayed action of citrate need be feared and no calcium need be administered. The other possible effect of the citrate, namely, to produce an alkalosis, would be expected to be salutary rather than harmful since an alkaline pH may tend to prevent serologic clumping and promote excretion of the products of hemolysis. One infant (case 15) died from pyemia, possibly as a result of infection introduced through the use of bank blood, and this is the only fatality that could conceivably be attributed to the treatment itself.

With regard to the technic of the procedure, the method used by us, besides being simple, is safe. We have had no operative mortality in a series of 40 transfusions performed to date. The use of heparin does not appear to be harmful or dangerous since it causes no tendency to bleed except from damaged or cut blood vessels and the heparin effect is nullified by the time the procedure is completed. Different methods of performing exchange transfusions have been suggested by other workers. With regard to the syringe method of Wallerstein,<sup>21</sup> this does not lend itself to the use of large amounts of blood except perhaps for operators with considerable experience and a high degree of technical skill. Some objections have also been raised to the use of the sagittal sinus as the avenue for withdrawing blood. The ingenious umbilical catheter method of Diamond<sup>22</sup> has been widely used and has been considered by some workers to be simpler than the method described by us. Recently, a modification of Diamond's original method has been devised whereby the catheter is inserted through an incision into the femoral vein at the groin instead of through the umbilical vessels.<sup>23</sup> The theoretic objection to the catheter method may be raised that it is a blind procedure and would appear to be tiring to the operators, since syringes must be continuously used to aspirate as well as to inject the blood. More important the method is somewhat dangerous since we

\* This is introduced slowly and always directly through the infusion cannula. It is never injected into the tubing.

know of at least two deaths from air-embolism that have occurred, and others in which death resulted from thrombosis and from hemorrhage into the peritoneal cavity. Also, technical failures have occurred even in experienced hands when the umbilical vessels could not be catheterized. Furthermore, the procedure usually cannot be carried out after twenty-four hours when the umbilical vessels close up. On the other hand, in our own series of over 40 exchange transfusions performed by using the radial artery for bleeding and the saphenous vein for the infusion, we have not had a single technical failure or operative mortality.

#### SUMMARY

1 In the authors' technic of exchange transfusion, citrated blood is introduced into the saphenous vein at the ankle and the infant's blood simultaneously with drawn from the radial artery at the wrist, coagulation being prevented by the administration of small amounts of heparin. The procedure besides being simple, is safe, there having been no operative mortality in more than 40 transfusions.

2 The results of exchange transfusion therapy in erythroblastosis in our first 28 cases are presented. Of these 28 cases, 16 were very severe and almost certainly would have been lethal if left untreated, 6 were of moderate severity, and 6 were mild. Only 7 of the infants died, and the available data indicate that the mortality would have been at least twice as high had the usual treatment with simple transfusions been given.

3 Aside from its greater efficacy in reducing mortality, exchange transfusion is more efficient, so that supplementary treatment is not required as a rule.

4 Fresh blood should be used instead of bank blood because of its greater survival time and smaller likelihood of introducing infection.

5 All infants who have survived have developed normally both physically and mentally and have shown no sequelae of liver or brain damage.

6 The most reliable index of the severity of the disease in the erythroblastic infant is provided by antenatal titrations of the maternal univalent Rh antibodies, as well as by tests for the presence of univalent antibodies in the infant's blood.

#### REFERENCES

- 1 WIENER, A. S. AND WEXLER, I. B. The use of heparin when performing exchange transfusions in newborn infants. *J. Lab. & Clin. Med.* 31: 1016-1019, 1946.
- 2 ———, AND GRUNDFAST, T. Therapy of erythroblastosis fetalis with exchange transfusion. *Bull. N. Y. Acad. Med.* 23: 207-220, 1947.
- 3 ———, AND SHULMAN, A. Therapy of severe erythroblastosis fetalis with repeated and massive exchange transfusions. *Am. J. Clin. Path.* 28: 141-151, 1948.
- 4 ——— AND ———. Antenatal selection of donors for exchange transfusion in erythroblastosis. *Ann. N. Y. Acad. Sci.* 296-302, 1948.
- 5 ———. Notes on the pathogenesis of congenital hemolytic disease (erythroblastosis fetalis). *Am. J. Clin. Path.* 16: 319-321, 1946.
- 6 ——— AND BRODY, M. The encephalopathy (kernicterus) of erythroblastosis fetalis: its serologic diagnosis and pathogenesis. *Am. J. Ment. Dis.* 51: 1-14, 1946.
- 7 ———. Rh factor in immunological reactions. *Annals of Allergy* 6: 293-305, 1948.
- 8 ———. Recent developments in the knowledge of the Rh-Hr blood types: tests for Rh sensitization. *Am. J. Clin. Path.* 16: 477-497, 1946.
- 9 ———, HURST, J. G. AND SONN-GORDON, E. B. Studies on the conglutination reaction with special reference to the nature of conglutinin. *J. Expt. Med.* 86: 267-284, 1947.

- <sup>10</sup> WITEESKY E, RUBIN M I AND BLUM, L. Studies in erythroblastosis fetalis I Activation of the incomplete Rh antibody by the serum of full term and premature newborn infants J Lab & Clin Med 32 1330-1338, 1947
- <sup>11</sup> KATSELY, M H, BLOCH E H, ELIOT T S AND WARNER L. Sludged blood, Science 106 431-440 1947
- <sup>12</sup> WIENER A S, AND GORDON E B. Studies on the conglutination test in erythroblastosis fetalis J Lab & Clin Med 33 181-188 1948
- <sup>13</sup> SACKS M S, KUHNS, W J, AND JAHN S.F. Studies in Rh immunization in pregnancy Am J Obst & Gynec 54 400-414, 1947
- <sup>14</sup> WIENER, A S, AND HURST J G. A new test (direct test) for Rh blocking antibodies Exper Med & Surg 5 284-298, 1947
- <sup>15</sup> ———. Diagnosis and treatment of anemia of the newborn caused by occult placental hemorrhage Am J Obst & Gynec 56 717-722 1948
- <sup>16</sup> ———. SONN-GORDON E B AND HANDMAN L. Heredity of the Rh blood types VI Additional family studies with special reference to the theory of multiple allelic genes J Immunol 57 203-210 1947
- <sup>17</sup> ———. A new test (blocking test) for Rh sensitization Proc Soc Exper Biol & Med 56 173-176 1944
- <sup>18</sup> ———. Conglutination test for Rh sensitization J Lab & Clin Med 30 662-667 1945
- <sup>19</sup> WEXLER I B, PINCUS J B, NATELSON S AND LUGOVY J K. The fate of citrate in erythroblastotic infants treated with exchange transfusion In preparation
- <sup>20</sup> WALLACE J T, WIENER, A S AND DOYLE M H. Rh sensitization in a primipara caused by intramuscular injection of human serum resulting in fatal erythroblastosis Am J Obst & Gynec in press
- <sup>21</sup> WALLERSTEIN, H. Substitution transfusion A new treatment for severe erythroblastosis fetalis Am J Dis Child 73 19 1947
- <sup>22</sup> DIAMOND L K. Replacement transfusion as a treatment for erythroblastosis fetalis Pediatrics 2 520 1948
- <sup>23</sup> ARNOLD, D P, AND ALFORD K M. A new technique for replacement transfusion in the treatment of hemolytic disease of the newborn infant J Pediatr 32 113, 1948
- <sup>24</sup> WIENER A S AND HANDMAN L. Simple method of detecting univalent antibodies in sera containing patent bivalent antibodies, with remarks concerning the pathogenesis of erythroblastosis fetalis Exper Med & Surg 6 416-427 1948



# RECENT STUDIES OF MULTIPLE MYELOMA STERNAL AND RIB PUNCTURE AND THE RESULTS OF TREATMENT WITH STILBAMIDINE

By SIMON PROPP, M D , L WHITTINGTON GORHAM, M D , AND SAMUEL KANTOR, M D

**M**ULTIPLE MYELOMA is commonly known as a malignant disease characterized by bone pain, deformity and abnormal fragility of the osseous system, cachexia, and Bence-Jones proteinuria. The tumors tend to be multiple. They are found most frequently in the spine, ribs, skull, bones of the shoulder girdle, pelvis, sternum and upper ends of the humeri and femora, where active blood formation occurs in the adult.

In 1845, Bence-Jones<sup>1</sup> found an unusual protein in the urine of a patient who complained of pain in the chest, back, and loins. This protein, which coagulated at 55 to 60 C. and redissolved upon boiling, has since been known by the name of the discoverer. Von Rustizky,<sup>2</sup> in 1873, first described a condition with multiple tumors of the bones which consisted of proliferating elements of bone marrow, under the title *Multiples Myelom*. Kahler<sup>3</sup> associated Bence-Jones proteinuria with multiple myeloma in 1889. The term *Kahler's disease* is frequently used as a synonym for this condition.

The pathology of multiple myeloma has been considered to be that of a neoplasm of the bone marrow in which the cytology varies depending upon the type of marrow cell involved. There is a diffuse proliferation of the malignant cells within the marrow. Arkinson<sup>4</sup> has summarized 643 cases of multiple myeloma. Of these 207 were classified as plasmacytoma, 27 myeloblastoma, 24 myelocytoma, 16 lymphocytoma, 5 erythroblastoma, 32 mixed, and 332 were unclassified. More recently, since the advent of the use of sternal marrow aspiration for diagnosis, reports on multiple myeloma have been almost entirely of the plasma cell type and there has been a definite trend to regard this disease as of plasma cell origin only.<sup>5, 6</sup>

The laboratory findings useful in diagnosis may be listed as follows: Bence-Jones proteinuria, hyperglobulinemia, excessive rouleaux formation of erythrocytes with clumping in Hayem's solution, and rapid sedimentation rate, osteoporosis by x-ray, hypercalcemia with normal or moderately elevated alkaline phosphatase and serum phosphorus values, anemia, myeloma cells\* in the peripheral blood, and myeloma cells in marrow aspiration. The latter finding has come to be regarded as pathognomonic of this disease. A positive marrow aspiration or surgical biopsy is necessary to establish the diagnosis.

The course of multiple myeloma is progressively fatal over a period of a few

From the Department of Medicine, Albany Medical College, Albany, New York.

Presented in part at the Western New York Regional Meeting of the American College of Physicians, Syracuse, New York, October 28, 1947.

Supported in part by a grant from the Winthrop Research Fund of the Albany Medical College.

\* The term myeloma cell in this article is used interchangeably with plasma cell to denote the series of dysplastic plasma cells observed in the bone marrow in multiple myeloma.

months to six years or longer from the time the diagnosis is made.<sup>5</sup> Treatment has been palliative. Roentgen therapy, nitrogen mustard, and radioactive phosphorus have shown no curative value. Snapper<sup>7, 8</sup> has introduced an interesting new chemotherapeutic agent in the treatment of this disease. He has apparently obtained relief of pain repeatedly in patients with multiple myeloma by the use of stilbamidine and pentamidine. Stilbamidine has proved very effective in treating visceral leishmaniasis. Because this disease and multiple myeloma are accompanied by hyperglobulinemia, it was reasoned empirically that stilbamidine might be of value in both. Kopac<sup>9</sup> has indicated that stilbamidine may act on nucleoproteins by demonstrating, *in vitro*, dissociation of protamine-ribonucleate complexes, the stilbamidine releasing protamine and simultaneously binding nucleic acids. Snapper has shown that following treatment of patients with multiple myeloma with stilbamidine, on a low animal protein diet, large basophilic inclusion bodies appeared in a high percentage of the myeloma cells. These were produced only in the presence of hyperglobulinemia or Bence-Jones proteinuria and appeared in 12 of 13 patients with increased blood globulin.<sup>10</sup> He has demonstrated that these inclusion bodies contained ribose nucleic acid, by studying the action of ribonuclease on the granules and by the use of the quartz microscope.<sup>11</sup> Stilbamidine was found by analysis in myeloma tissue obtained at postmortem examination eight days after completion of a course of this drug. He has advanced the theory that stilbamidine reacts with the cytoplasmic nucleoproteins of myeloma cells only, and not with nucleoproteins of other cells. A high protein diet, according to Snapper's theory, interferes with this reaction. He has suggested that pain is relieved because myeloma cell proliferation is arrested. Lack of expansion of osteolytic lesions for some time after cessation of treatment was demonstrated in some patients. This occurred despite the fact that the percentage of myeloma cells in the marrow smears was not shown to decrease. No changes in the myeloma cells were found after treatment with pentamidine, although relief of pain was obtained.

A feeling of formication about the face was common accompanying the injections of stilbamidine. A high incidence of dissociated anesthesia of the trigeminal nerve occurred two and a half to five months following treatment. This caused considerable distress in the form of severe persistent itching in only one of Snapper's patients. In most cases the discomfort gradually subsided. No toxic effects were produced on the liver or hematopoietic system. Renal failure was precipitated in two cases and caution was advocated in treating patients with renal damage and insufficiency.<sup>1</sup>

Ten out of eleven patients treated only with stilbamidine by Snapper were relieved of pain.<sup>12</sup> Pentamidine was used successfully in the one case in which stilbamidine failed. In two cases pentamidine was ineffective but subsequent use of stilbamidine caused the pain to disappear. Recurrence of pain required repetition of stilbamidine treatment and it was emphasized that although progress of the disease was temporarily checked no cure was obtained.

This article deals with a study of six cases of multiple myeloma admitted to the Albany Hospital within a relatively short period of time. An opportunity was presented to study myeloma cells before and after treatment with stilbamidine, and to observe the clinical effects of this drug.

## METHODS

Each case received a complete hospital work-up with appropriate laboratory and x-ray studies. Sternal marrow aspiration was done in all cases before and after treatment with stilbamidine. In addition, rib marrow punctures\* were performed on four occasions. Because of the great dependence placed on marrow puncture in the diagnosis of multiple myeloma, a more elaborate technic was developed which differed somewhat from that previously described.<sup>14</sup> Changes in procedure consisted in using heparin solution and 1 cc syringes for marrow aspiration. Heparin solution containing ten units per 1 cc † was used in sufficient amount to wet the syringe only. This syringe was then utilized to aspirate 0.2 cc of marrow fluid. The heparin prevented clotting, and subsequent steps could be carried out at the leisure of the operator. Coverslip smears were made directly from the marrow fluid without mixing. A cresyl blue wet smear and two supravital smears were then made. The fluid remaining was ejected onto a hollow slide. Marrow bits were identified by tilting the slide and these were picked up with pipets and smears repeated. The marrow mixture was then placed in a Wintrobe hematocrit tube, centrifuged, and a third set of preparations made from the buffy layer. The dry coverslip smears were subsequently stained with Wright's stain and a peroxidase-Wright's stain was done on a selected smear. This method of procedure gave three possibilities of securing a representative sample of marrow cells, and precluded the chance of missing the diagnostic picture from chance selection of material for smears, dilution with sinusoidal blood, and rapid clot formation in the aspirating syringe.

Rib puncture was performed in the scapular line. The skin and periosteum were anesthetized with procaine in a manner similar to that employed in sternal puncture. The margins of the rib were grasped between the thumb and index finger to ascertain the rib center. A sternal puncture needle 1.5 cm or less in length was used because of the possible danger of entering the pleural cavity. ‡ The center of the rib was bored with a rotary motion of the needle and the marrow cavity entered in a fashion similar to sternal puncture. The same preparations of marrow smears were made as enumerated above §.

\* Rib marrow puncture was first performed on one case suspected of rib malignancy immediately post mortem. The procedure was so simple and the results so satisfactory that it was performed later on a patient diagnosed clinically as multiple myeloma when sternal puncture was unsuccessful. A second sternal puncture and the rib puncture which was done in an area termed pathologic by the roentgenologist proved to contain normal bone marrow. The patient subsequently recovered. Since then rib punctures have been performed on sixteen patients and excellent marrow preparations obtained. A requisite for rib puncture is the careful palpation of the selected rib. Puncture should never be done if the rib is not easily palpable.

† Lilly's solution of sodium heparin.

‡ It was found by puncturing ribs during post mortem examinations that if considerable force were exerted the tip of a 1.5 cm needle could be forced through the parietal pleura of a thin person. A 1 cm needle could not be made to penetrate completely through the rib. A very definite give was usually experienced when the outer thin bony plate of a rib was pierced and the marrow cavity entered. This sensation was not invariably felt however so that aspiration was always attempted when the needle was firmly fixed in the bone. Then the needle was slowly advanced until marrow fluid was obtained or the sensation of entering the marrow cavity experienced.

§ Comparison of rib and sternal preparations have shown a similar marrow picture in 13 instances when both were done immediately following each other. Variations in amount of marrow material

The cases of multiple myeloma were treated with stilbamidine according to Snapper's method, as follows. The stilbamidine was dissolved in 10 cc of sterile distilled water and used immediately. Injections were given intravenously, starting with a dose of 50 mg. One hundred mg were given the following day, and then 150 mg daily for a total of 20 treatments which constituted the usual course. Atropine sulfate Gr 1/150 was given hypodermically 30 minutes before each injection to prevent or minimize immediate vasomotor reactions as recommended by Snapper. The total dosages of stilbamidine and the diets given to each patient are listed in table 2.

The clinical and laboratory findings and therapeutic results are illustrated in the following case histories.

#### CASE I

A S, a 61 year old white male office worker, was admitted to the Albany Hospital on Jan 7 1947. He had been ill for 3 months with unexplained fever. Two weeks before his hospital admission he developed pain in his left chest which was aggravated by cough and deep breathing. Physical examination revealed a fever of  $101^{\circ}$  and evidence of pneumonia in the left lower lobe which was confirmed by x-ray. The liver was enlarged and tender. The heart had irregular rhythm and there was a loud blowing apical systolic murmur. An electrocardiogram revealed auricular fibrillation and low T waves with slurring of QRS in the standard leads indicating myocardial damage. Hemoglobin was 7.5 Gm, red blood cells 2,000,000, white blood cells 8,150, segmented neutrophils 80 per cent, lymphocytes 14 per cent, and monocytes 6 per cent. Erythrocyte sedimentation rate Wintrobe was 10 mm in one hour.

The patient's pneumonia was treated effectively with penicillin and three 500 cc blood transfusions. He developed a nonpurulent, sterile, pleural effusion. Abnormal clumping of erythrocytes was noted on routine blood counting and because of this finding he was studied for multiple myeloma. The urine was positive for Bence Jones protein and showed a 1 plus albumin. Serum protein was 11.5 Gm per cent total with albumin 1.6 and globulin 9.9 Gm per cent, an A-G ratio of 0.16. Serum calcium was 11.9 mg per cent, phosphorus 3.2 mg per cent, alkaline phosphatase 3.9 Bodansky units, and NPN 60 mg per cent. X-ray examinations of the skull and ribs were normal. Sternal puncture revealed marked replacement of normal marrow elements by plasma cells. These made up 91 per cent of a 500 white cell differential count. This established the diagnosis of multiple myeloma.

Treatment was carried out with stilbamidine. Eighteen injections totaling 2.7 Gm were administered. Because of the poor nutritional state of the patient no restriction of protein was ordered. Unpleasant effects of the injections consisted of transient prickly feelings about the mouth, eyes and ears at the time of treatment and recurrent nausea and vomiting. These manifestations were not severe. Sternal puncture following the course of stilbamidine showed that 34 per cent of cells in the marrow smears were of the myeloma type. Azurophilic granulation was noted in the cytoplasm of some of these cells but no basophilic inclusion bodies were found.

The patient was discharged without improvement in his general condition. He subsequently expired on May 15 1947, 3 months from the time his treatment was concluded. No autopsy was obtained.

#### COMMENT

The diagnosis of multiple myeloma was only suspected in this case because of the abnormal clumping of erythrocytes noted in Hayem's solution in a routine blood count. His first presenting symptom of unexplained fever without pain was atypical. Confirmatory laboratory findings of hyperglobulinemia and Bence-Jones proteinuria were offset by negative x-ray examinations of skull and ribs. Diagnosis was established by sternal puncture. Treatment with stilbamidine without pro-

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however have been observed. Further studies on rib puncture as a procedure to complement or supplement sternal puncture are being made.

tein restriction failed to effect a remission in the course of the disease or to produce basophilic granulation in the cytoplasm of the cells although hyperglobulinemia was present

# CASE 2

R. L., a 58 year old Italian male, was admitted to the Albany Hospital on January 28, 1947 with a chief complaint of vertigo. For ten days prior to admission he had repeated, transient attacks of dizziness and weakness and for six months had suffered from generalized headaches. There was a weight loss of 15 pounds during this period. He had had an acute infection in the right ear three weeks before admission,



FIGS 1-3 X RAYS OF SKULL, CASE 2, R. L.

FIG 1 January 29 1947 before treatment with stilbamidine

which had subsided. Examination revealed moderate tenderness over the left temporal region anterior to the ear. The Romberg test was strongly positive with the patient falling to the right. Blood pressure was 160 mm. of mercury systolic and 100 mm. diastolic. A routine x-ray of the skull showed numerous small punched-out areas of decreased density (fig 1). Further studies of the osseous system revealed evidence of active bone destruction in the 5th lumbar vertebra and some compression of the second and third lumbar segments. Laboratory examinations showed hemoglobin 13 grams, red blood cells 4,400,000, white cells 10,000 with a normal differential count, blood Wassermann negative, urine normal, total serum protein 12.1 Gm per cent, albumin 3.7 Gm per cent, and globulin 8.4 Gm per cent with an A-G ratio of 0.4. Serum calcium 10.5 mg per cent, phosphorus 3.8 mg per cent, alkaline phosphatase 3.6 Bodansky units, NPN 39 mg per cent, creatinine 0.9 mg per cent. No Bence Jones protein was found on repeated tests. Sternal puncture preparations contained 19.5 per cent plasma cells.

The patient was given a course of 20 injections of stilbamidine totaling 2.85 Gm. The diet was not restricted. He complained of burning of the skin, lacrimation, salivation, bilateral tinnitus and restlessness as an immediate reaction to the drug administration. These complaints subsided within a few minutes following the injections. Four weeks following treatment a sternal puncture revealed a reduction of myeloma cells to 6.2 per cent. The majority of these cells, 83.8 per cent, showed large basophilic inclusion bodies in the cytoplasm which were identical with those described by Snapper.<sup>8</sup> A roentgenogram of the skull indicated a definite increase in the areas of decreased density (fig. 2). Subjectively the patient felt generally improved, and the headaches and dizziness were relieved. Five weeks after completion of his first course of stilbamidine he had a recurrence of severe generalized headaches and aching pain in the



Fig. 2. April 3, 1947, five weeks after completion of first course of stilbamidine

lumbar spine. A second course of the drug, consisting of 1.35 Gm, was given over a period of ten days with the patient on a low animal protein diet. Relief of symptoms occurred and he returned to light work. Seven weeks after this course of treatment he complained of numbness about the mouth involving most of the face. No neurologic changes were noted. Later, intense burning in this region occurred, particularly at night. This still persisted after six months of observation. Rib puncture on Sept. 9, 1947, six and one half months following his first treatment and four and one half months after his second course of stilbamidine, revealed 58.8 per cent myeloma cells. Basophilic inclusion bodies were still present in 54 per cent of the cells. There were 2.3 per cent plasmablasts. Skull x-ray at this time showed further increase in the osteolytic lesions (fig. 3).

#### COMMENT

The clue to diagnosis in this case was obtained from an x-ray of the skull taken because of the patient's complaints of vertigo and headaches. The possibility of

multiple myeloma had not been previously entertained. Further osseous lesions in the lumbar vertebrae and hyperglobulinemia were confirmatory evidence. Sternal puncture established the diagnosis of multiple myeloma.

Treatment with stilbamidine without restriction of animal protein not only produced a remission of symptoms but also caused typical basophilic cytoplasmic inclusions in the majority of myeloma cells. This occurred at a time when osteoporotic lesions in the skull were increasing in size. A severe persistent trigeminal neuropathy followed a second course of treatment. Basophilic inclusion bodies



FIG. 3 September 4, 1947: six months after first course and four months after second course of stilbamidine.

were observed in the myeloma cells obtained from rib puncture four and one half months after treatment was concluded.

### CASE 3

C. S. H., a 47-year-old white male was admitted to the Albany Hospital on Feb. 26, 1947. He had been ill for 9 months with pain in his ribs, progressive weakness and fatigue, loss of weight and failing vision. He was told that he was anemic four months before admission to the hospital. Examination revealed a pale, thin patient who appeared chronically ill. There was tenderness to pressure over the lower ribs bilaterally. There was a soft, blowing apical cardiac murmur. The liver edge was palpable two fingers below the costal margin. Ophthalmic examination revealed presbyopia only.

*Laboratory studies* Positive test for Bence Jones protein and 4 plus albuminuria, hemoglobin 10 Gm, red blood cells 3,320,000, white blood cells 9,300 with a normal differential count, Wintrobe sedimentation rate 12 mm in one hour blood Wassermann negative total serum protein 5.6 Gm per cent with an A-G ratio of 2.5 NPN 30 mg per cent calcium 10.4 mg per cent phosphorus 3.2 mg per cent and alkaline phosphatase 4.2 Bodansky units X-rays of the skull and ribs were reported as being normal. A second roentgenogram of the ribs showed a suggestion of metastatic tumor on the lower left. Sternal puncture revealed that 23 per cent of marrow cells were of the large plasma cell type.

The patient was placed on a low animal protein diet and given a course of stilbamidine totaling 2.85 Gm. He also received two 500 cc blood transfusions. Sternal puncture was repeated after 2.55 Gm of stilbamidine or 18 injections had been administered. The marrow contained 43.4 per cent myeloma cells of which the great majority (82.9 per cent) contained basophilic inclusion bodies. At the conclusion of treatment hemoglobin was 9.5 Gm, red blood cells 4,160,000 and white blood cells 6,800 with a normal differential count.

This patient improved considerably. The pain in his ribs subsided, his vision improved and he gained in strength. He returned home and reported in six weeks by letter that he felt fine. Follow up of this patient revealed that two months after treatment he was relieved of pain and felt well except for a general skin rash and a sore tongue. His doctor reported that there was, however, a progressive decline in his general state. Retreatment was advised but the patient had moved away and the referring doctor was unable to locate his new residence. A follow up obtained by letter, however, written on Dec. 25, 1947, indicated that the patient was still ambulatory, but otherwise totally incapacitated by his illness.

#### COMMENT

This patient's symptomatology fitted the clinical picture of multiple myeloma. Bence-Jones proteinuria and anemia were the positive laboratory findings and x-rays were negative. There was no hyperglobulinemia. Sternal puncture findings were pathognomonic.

Stilbamidine treatment with a low animal protein diet plus transfusions improved this patient symptomatically and typical basophilic inclusion bodies were found in the majority of the myeloma cells, although the relative percentage of the tumor cells had definitely increased in the marrow. The treatment did not, however, have any sustained effect upon his general condition.

#### CASE 4

A W., a 61 year old male office manager, was admitted to the Albany Hospital on Jan. 12, 1947, with the chief complaint of an infection in his nose of one week's duration. He was a known diabetic who had been well controlled with diet and insulin for 30 years.

Examination revealed impetiginous lesions of the nose and forehead, a blood pressure of 170 mm. of mercury systolic and 80 mm. diastolic, and a palpable liver felt three fingers below the costal margin.

Urinalysis was normal except for a trace of sugar. Blood hemoglobin was 10 Gm, red blood cells 2,930,000, white blood cells 6,900, segmented neutrophils 71 per cent, lymphocytes 27 per cent, monocytes 2 per cent. NPN was 38 mg per cent and the blood Wassermann was negative. Total protein taken for investigation of liver function, was 10.7 Gm per cent with albumin 2.1 Gm per cent and globulin 8.6 Gm per cent, an A-G ratio of 0.24. Because of the hyperglobulinemia further studies were done. Urine positive for Bence Jones protein, clumping of erythrocytes was observed in Hayem's solution, and Wintrobe sedimentation rate was 61 mm. in one hour. Sternal puncture on Jan. 23, revealed 14 per cent large plasma cells and a marked reduction in erythroid and myeloid cells. A second sternal puncture at a higher level showed similar findings.

A diagnosis of multiple myeloma was made but because the patient was asymptomatic he was discharged from the hospital. Five months later he was readmitted because of a severe vaccinia. At this time, he complained of pain in his right hip but x-ray examination of his pelvis was normal. He was again discharged but shortly afterward began to have severe pain in the left chest in the region of the



fifth to the ninth ribs. He also suffered from diplopia. X-ray examination at this time showed evidence of bone destruction in the ribs. He was readmitted on July 17, 1947, for treatment with stilbamidine because of persistent bone pain. Examination revealed weakness of the left external rectus ocular muscle, with inability to move the left eye laterally. The liver was still enlarged. The urine contained 2 to 3 plus albumin but no Bence Jones protein. Hemoglobin was 9.5 Gm, red blood cells 3,200,000, white blood cells 6,300 segmented neutrophils 71 per cent, NPN was 42 mg per cent, total protein 9.1 Gm per cent, with albumin 2.2 Gm per cent and globulin 6.9 Gm per cent, an A-G ratio of 0.32.

The patient received a course of 20 injections totaling 2.85 Gm of stilbamidine from July 18 to Aug. 9. The diet contained 2,600 calories with 97 Gm of protein which was qualitatively unrestricted. Immediate reactions to the treatment were a burning about the mouth sometimes extending into the eyes, which was only momentary and nausea and at times vomiting which were delayed until later in the day. The diplopia and bone pain were unrelieved. Sternal and rib puncture on Aug. 12, showed a myelophthisic marrow with 61.2 per cent myeloma cells. No basophilic inclusion bodies were observed. The patient was then placed on a low animal protein diet and a total of 1.5 Gm of stilbamidine was administered in a course of ten injections from Aug. 15 to 27. There was no relief of pain. Puncture of the right eighth rib was performed at this time and no change in the marrow picture was found. There were no basophilic inclusion bodies in the myeloma cells.

On Sept. 10, 1947, a prefrontal lobotomy was performed for relief of pain. The patient expired post-operatively.

Autopsy confirmed the diagnosis of multiple myeloma. Infiltrations of plasma cells were noted in the liver.

#### COMMENT

The diagnosis of multiple myeloma was made during the hospitalization of this patient for impetigo and diabetes mellitus, because of the presence of hepatomegaly. The findings of hyperglobulinemia, Bence-Jones protein, clumping of erythrocytes in Hayem's solution and positive sternal puncture complete the diagnostic picture although the patient had no symptoms of the disease and x-ray studies of the bones were negative.

Treatment with a full course of stilbamidine without a low animal protein diet failed to relieve pain or effect the myeloma cells. A second course of 1.5 Gm of stilbamidine on a low animal protein diet also did not alleviate pain or produce basophilic inclusion bodies in the myeloma cells. Failure of treatment thus occurred despite the presence of both hyperglobulinemia and Bence-Jones proteinuria.

#### CASE 5

A. V., a white farmer, 72, was admitted to the Albany Hospital on Aug. 5, 1947, because of pain in the back, left hip and left leg of six months duration. The pain in the back and left hip occurred after a fall. About one month before admission the pain began to radiate down the medial aspect of the left leg. The pain was intermittent, sharp and worse at night. The patient also had frequent nose bleeds since the onset of his illness.

Physical examination showed emaciation. The skin was dry and loose. Blood pressure was 158 mm of mercury systolic and 70 mm diastolic. There was tenderness over the fourth right rib. The liver was enlarged and could be palpated two fingers below the costal margin. There was diminished sensation to light touch along the medial aspect of the left thigh and calf. The left patellar reflex was reduced.

The urine contained 3 plus albumin and many hyaline casts but no Bence Jones protein. Hemoglobin was 8.5 Gm, red blood cells were 2,860,000 and white blood cells were 10,150. On Aug. 6, the white blood cells were 12,200 with segmented neutrophils 48 per cent and lymphocytes 52 per cent. The blood Wassermann was negative. Total plasma protein was 12.7 Gm per cent with albumin 2.3 Gm per cent and globulin 10.4 Gm per cent, an A-G ratio of 0.22. Serum could not be obtained and clot retraction could not be studied because the blood rapidly formed a gel and no fluid portion remained. The clotting time was one minute and 18 seconds, coagulation time three minutes and platelet count 157,000. Clumping

of erythrocytes occurred in Hayem's solution. X rays revealed many small oval areas of lessened density throughout the skull and in the upper third of the left humerus. Myelogram showed bilateral deformities opposite lumbar vertebrae 3 and 4 more marked on the left which was consistent with a displaced disc. Spinal fluid contained 60 mgs per cent protein and the Wassermann test was negative. Sternal and rib punctures on Aug. 9 were diagnostic of multiple myeloma. There were 45.6 per cent plasma cells. Of these 1.2 per cent were plasmablasts, and 4.8 per cent were young forms. There was great variation in size and appearance of the cells. Syncytial sheets of small cells with nuclei placed centrally, or nearly so and uniformly basophilic cytoplasm were seen which resembled basophilic normoblasts. Larger cells had typically eccentric nuclei and abundant basophilic cytoplasm characteristic of plasma cell myeloma. Subsequently a few large plasma cells of similar type were observed in the peripheral blood smear.

The patient's condition deteriorated rapidly. On the third hospital day he was given 500 cc. of blood by indirect transfusion. On the sixth day he became drowsy, stopped taking fluids and then gradually became comatose. NPN was 72 mg per cent. On Aug. 15, NPN was 58 mg per cent, and plasma  $\text{CO}_2$  43 volumes per cent. Fifty mg. of stilbamidine were given intravenously, and 100 mg. the following day. The patient expired in coma on the eleventh hospital day.

Autopsy revealed involvement of lumbar vertebrae, skull and sternum with plasma cell myeloma. An unusual phenomenon was the finding of a complete cast of the heart and larger vessels formed by a firm gel composed of blood plasma.

### COMMENT

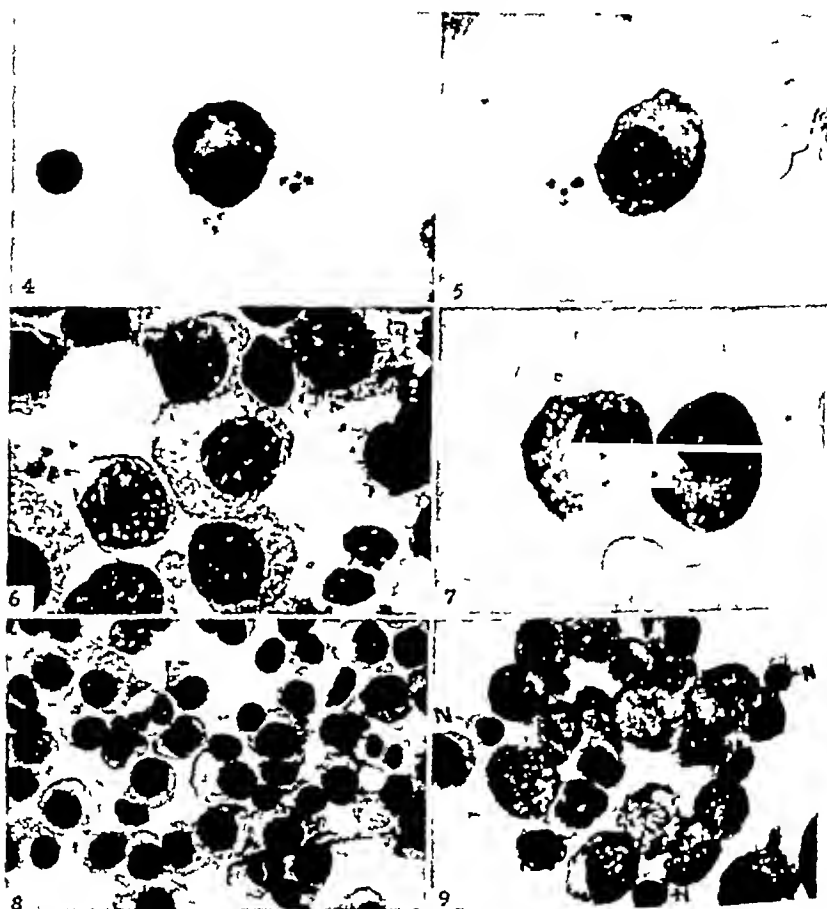
This was a typical, malignant type of multiple myeloma showing a fully developed symptomatic and pathologic picture. Sternal and rib puncture confirmed the diagnosis. The marrow cytology was interesting because of the presence of small myeloma cells resembling basophilic normoblasts. Treatment with stilbamidine was attempted only because of the obviously bad prognosis. Autopsy confirmed the diagnosis and a striking finding was the presence of a cast of the heart and vascular system consisting of a firm gel of the blood plasma.

### CASE 6

E. J. B., an 85 year old single white female, was admitted to the Albany Hospital on Nov. 22, 1947. She had complained of pain in her back in the region of the lower ribs which radiated anteriorly about her chest, for four months. The pain was almost constant but varied in severity. It was aggravated by motion. She had lost weight and strength and for the month prior to admission, had suffered from anorexia, dyspnea and recurrent vomiting. Physical examination showed a very thin, dehydrated patient who was tender over the lower thoracic vertebrae. The heart was enlarged and systolic murmurs were heard over the aortic and mitral areas. The clinical impression was osteomalacia or metastatic malignancy and arteriosclerotic heart disease.

The urine showed only a trace of albumin and tests were negative for Bence Jones protein. Hemoglobin was 8.0 Gm., red blood cells 2,650,000, white blood cells 8,750, segmented neutrophils 71 per cent, band neutrophils 1 per cent, eosinophils 1 per cent, basophils 1 per cent, and lymphocytes 26 per cent. Wintrobe erythrocyte sedimentation rate was 66 mm. in one hour. The blood Wassermann was negative. Serum phosphorus was 2.2 mg per cent, alkaline phosphatase 2.1 Bodansky units, serum calcium 11.3 mg per cent, and NPN 30 mg per cent. Total serum protein was 7.7 Gm. per cent of which albumin was 3.2 and globulin 4.5. An A-G ratio of 0.7. X ray examinations revealed a partial collapse of the bodies of thoracic vertebrae 7, 10, 11 and 12 with marked atrophic changes in all the vertebral bodies. There were multiple small areas of localized bone destruction throughout the ribs and in both scapulae. The 6th rib was fractured in the axillary line on the left. There were multiple minute areas of lessened density distributed throughout the skull. Sternal and rib aspirations were performed on Nov. 26 and the marrow smears revealed 25.4 per cent plasma cells and 0.4 per cent plasmablasts. This established the diagnosis of multiple myeloma. The patient was placed on a low animal protein diet and given a course of 12 injections of stilbamidine totaling 1.65 Gm. There was no reaction to the drug. She continued to have constant severe pain in the back and nausea and vomiting. Her general condition gradually grew worse and death occurred

on the twenty-second hospital day Autopsy revealed multiple myeloma of the plasma cell type involving the ribs sternum and vertebrae Smears of the sternal marrow obtained post mortem showed 21.4 per cent plasma cells and 0.4 per cent plasmablasts No basophilic inclusion bodies were observed



FIGS 4-15 PHOTOMICROGRAPHS OF MARROW SMEARS PREPARED FROM STERNAL AND RIB ASPIRATIONS  
(WRIGHT'S STAIN UNLESS OTHERWISE SPECIFIED)

- FIG 4 (upper left) Usual type of plasma cell from normal marrow ( $\times 1130$ )  
 FIG 5 (upper right) Large type of plasma cell from normal marrow ( $\times 1130$ )  
 FIG 6 (center left) Myeloma cells from Case 1, A, S ( $\times 1130$ )  
 FIG 7 (center right) Myeloma cells from Case 2, R, L ( $\times 1130$ )  
 FIG 8 (bottom left) Myeloma cells replacing normal marrow cells Case 1, A, S ( $\times 400$ )  
 FIG 9 (bottom right) Dysplastic plasma cells (myeloma cells) resembling normoblasts N=normoblasts All other cells are myeloma cells Case 5, S, V ( $\times 730$ )

#### COMMENT

This patient had multiple myeloma at the extreme age of 85 years The diagnosis was indicated by the clinical picture, x-ray findings, and hyperglobulinemia, and

was confirmed by marrow aspiration. Treatment with 1.65 Gm of stilbamidine failed to relieve pain or produce basophilic granulations in the myeloma cells, although hyperglobulinemia was present.

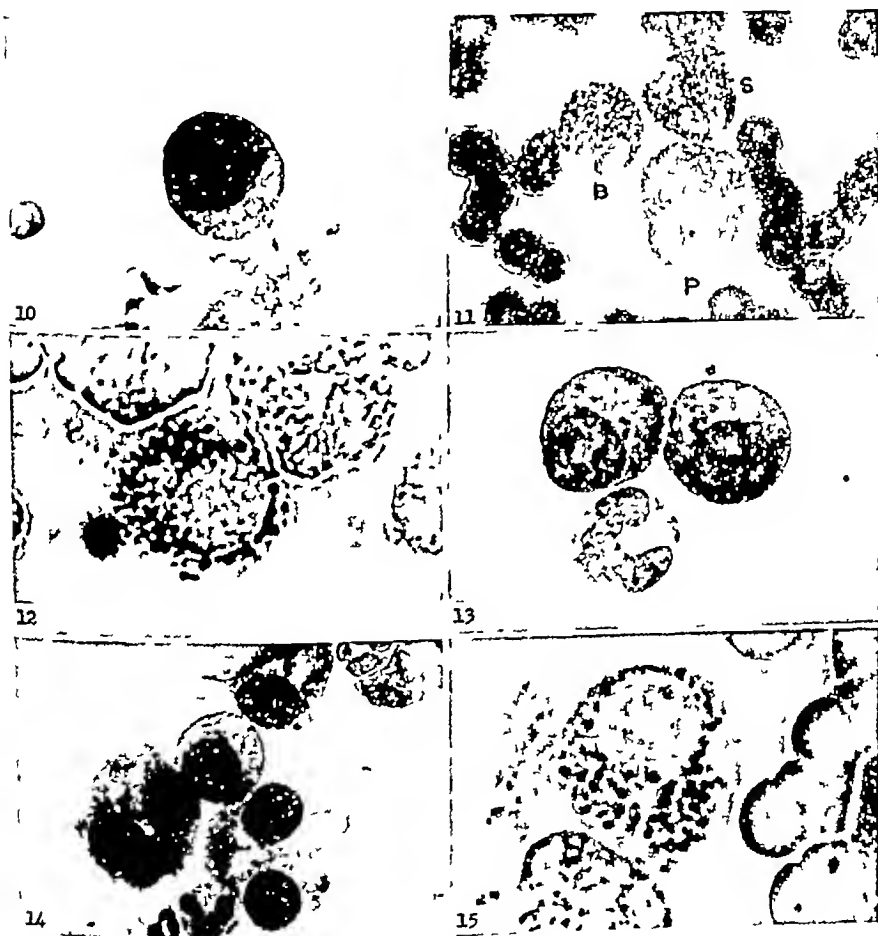


FIG 10 (upper left) Plasmablast from Case 5 S V ( $\times 1130$ )

FIG 11 (upper right) Peroxidase-Wright's stain from Case 2, R L P plasma (myeloma) cell S-segmented neutrophil B-band neutrophil ( $\times 1130$ )

FIG 12 (center left) Supravital stain of myeloma cell from Case 2, R L ( $\times 1230$ )

FIG 13 (center right), 14 (lower left) Myeloma cells after stilbamidine treatment Case 2 R L ( $\times 1130$ )

FIG 15 (lower right) Supravital stain of myeloma cell after stilbamidine treatment Case 2 R L ( $\times 1230$ )

#### CYTOLOGY

The cytology of multiple myeloma has recently been described by Diggs and Sirridge.<sup>6</sup> Their findings were based on fifty-five cases of plasma cell myeloma. Support was given to the thesis that multiple myeloma is derived from plasma

cells arising from primitive reticulum as a specific strain of cells. The term myeloma cell was objected to because it inferred a specific type of cell peculiar to multiple myeloma only. Our observations on the cytology of the six cases being reported were similar to those of Diggs and Sirridge.

Our cases were entirely of the plasma cell type. In general, with Wright's stain, the following characteristics were noted. The cells were oval in shape, the nuclei eccentric, round and pachychromatic but not typically cart-wheel or Radkern. Variation in size occurred not only in the various patients but also in each individual case. The cytoplasm was abundant and minor differences in intensity of its basophilic substance were present in the different cases. Vacuolization of the cytoplasm was common, and a perinuclear clear zone was a prominent feature. In case 5, small cells with uniformly basophilic cytoplasm and centrally placed nuclei were noted, which resembled basophilic normoblasts (fig. 9). The usual type of plasma cell in normal marrow is shown in figure 4. A second type of plasma cell with more abundant, less deeply basophilic cytoplasm, without vacuoles and with a larger nucleus, observed in the same marrow smear, is illustrated in figure 5. The latter closely resembles the typical cells found in our cases of multiple myeloma (figs. 6, 7). Plasmablasts of large size with a definitely eccentric, large leptochromatic nucleus containing nucleoli, and comparatively little, basophilic cytoplasm, were observed in small numbers (fig. 10). The highest percentage of plasmablasts was 2.3, seen in case 2. Young plasma cells containing large, relatively immature nuclei without nucleoli, and more abundant cytoplasm than the blast form, constituted 4.8 per cent of the myeloma cells in case 5. Very large cells with multiple separate nuclei were seen in all cases. Mitotic figures were not numerous. Sheets of plasma cells were commonly observed in preparations made from marrow bits and occasionally in direct smears of unconcentrated marrow fluid (fig. 8). The plasma cells were uniformly peroxidase negative (fig. 11).

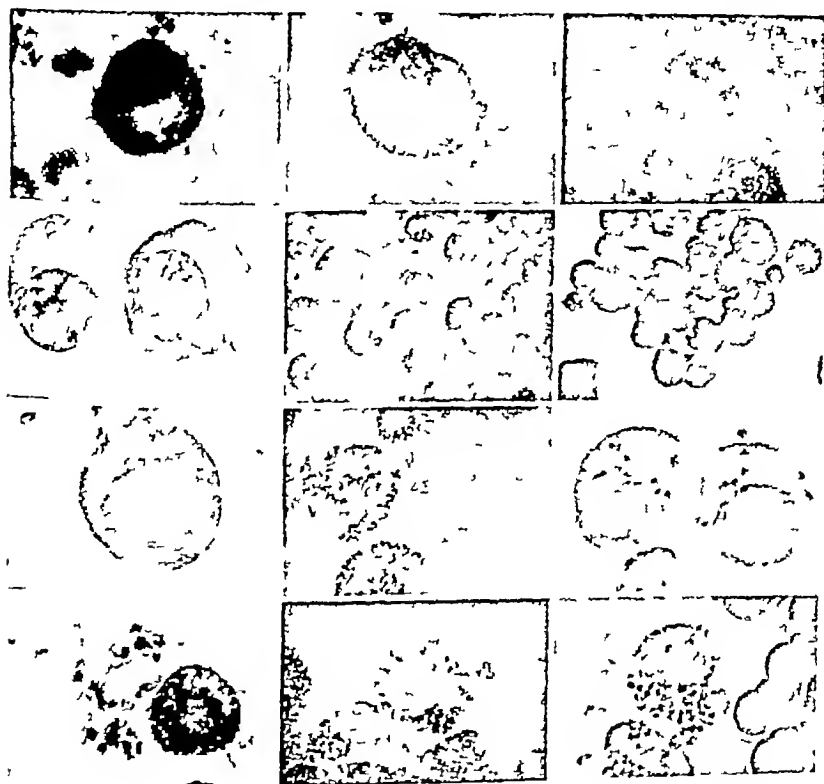
Supravital studies revealed a close similarity between the myeloma cells and large plasma cells seen in marrow smears made from patients with other conditions, and from normal marrow. The cells were large and oval, or round, with abundant cytoplasm and a distinct cell membrane. The nucleus was round and very definitely eccentric. Neutral red vacuoles of variable size were present external to the nucleus. These could be observed to enlarge as the preparations aged. Large mitochondria were a striking feature.<sup>18</sup> These were interspersed in the cytoplasm but were more abundant near the nucleus (fig. 12). The usual type of plasma cell seen in normal marrow was smaller and the mitochondria and neutral red vacuoles appeared to be less numerous and of smaller size.<sup>18</sup> However, a large type plasma cell was observed in one case of chronic aleukemic myelogenous leukemia treated with x-ray, which was indistinguishable from the typical plasma cell seen in the multiple myeloma patients.

After stilbamidine treatment, large basophilic inclusions were noted in the cytoplasm in the majority of the plasma cells stained with Wright's stain, in cases 2 and 3, (fig. 13, 14), while no inclusions were seen in the other four cases. No change was observed in cells stained by the peroxidase method. Although the

illustrated cell (fig 15) shows an increased size in neutral red vacuoles by supravital stain, this was not remarkable as compared to studies made prior to treatment

### DISCUSSION

The method of diagnosis of multiple myeloma in the cases reported is illustrated in table 1. The seven findings which are more pertinent to the diagnosis are listed



PHOTOMICROGRAPHS IN COLOR TAKEN FROM THE SAME MARROW SMEARS AS THE CORRESPONDING  
PHOTOMICROGRAPHS WITHOUT COLOR

Top row figs 4 5 6 Second row figs 7 8 9 ( $\times 450$ ) Third row figs 10 ( $\times 1410$ ) 11 13  
Fourth row figs 14 ( $\times 1000$ ) 12 ( $\times 750$ ) 15 ( $\times 750$ )

together and collateral findings which are common to other conditions are placed in the lower section of the chart. The table shows in striking fashion an observation that is well-known, that the clinical picture of multiple myeloma is extremely variable. The only constant feature in all cases was the presence of a positive marrow aspiration. In one patient (case 3), the diagnosis was made by sternal puncture although only bone pain, Bence-Jones protein, and anemia were present. A clinical diagnosis was made prior to laboratory or x-ray studies in two cases. The clue to the diagnosis was found by the simple observation of clumping of erythrocytes

in Hayem's solution in one instance, by x-ray of the skull because of vertigo and headaches in a second, and from the finding of hyperglobulinemia while investigating the presence of hepatomegaly in a third case

The great value of marrow aspiration in the differential diagnosis of multiple myeloma makes a satisfactory technic for this procedure extremely important. A method has been described which utilized unconcentrated marrow fluid, selected marrow bits, and a concentration of marrow cells obtained by centrifugation. The selection of marrow bits produced the most satisfactory marrow smears. Rib puncture was used successfully to complement and supplement sternal aspiration. This was found to be a simple and usually painless procedure. One important

TABLE 1—*Diagnosis of Multiple Myeloma*

	Case number					
	A <sup>1</sup> S	R <sup>2</sup> L	C <sup>3</sup> H	A <sup>4</sup> W	S <sup>5</sup> V	E <sup>6</sup> B
Bone pain	—	—*	+	—†	+	+
Osteoporosis	—	+	—	—	+	+
Bence Jones Protein	+	—	+	+	—	—
Hyperglobulinemia	+	+	—	+	+	+
Clumped RBC in Hayem's	+	—	—	+	+	—
Myeloma cells in blood	—	—	—	—	+	—
Marrow aspiration	+	+	+	+	+	+
Anemia	+	—	+	+	+	+
Albuminuria	+	—	+	+	+	+
Elevated NPN	+	—	—	+	+	—
	(60)	(39)	(38)	(38)	(72)	(30)
Hypercalcemia	—	—	—	Not done	Not done	—
Rapid RBC sedimentation	—	Not done	—	+	Not done	+
Autopsy	None	None	None	+	+	+

\* Patient had severe headaches with vertigo. Back pain occurred later.

† Severe rib pain developed later in the course of his disease.

advantage is psychologic, the patient being unable to observe the details of the puncture. Caution must be used and no rib puncture should be done on a patient in whom the outlines of the rib are not definitely palpable.

The criteria upon which a diagnosis of multiple myeloma is made from marrow aspiration are not well defined. The number of plasma cells in the preparations is variable and reports have been as low as four per cent in one of the cases of Diggs and Sirridge<sup>6</sup> and three per cent in the series of Rosenthal and Vogel.<sup>17</sup> In normal marrow the percentage of plasma cells is usually less than 1 per cent. However, in other conditions, they may be present in greater numbers. \* It is felt that the con-

\* In our own studies a marrow of fatal agranulocytosis showed practically a complete replacement with plasma cells and lymphocytes.

tent of plasma cells in the marrow in diseases other than multiple myeloma may not have been adequately studied. A factor which can not be evaluated is the admixture of sinusoidal blood in preparations made directly from aspirated marrow.\* It is believed that the marrow picture is pathognomonic when the predominant cell type is the myeloma cell as described above. The presence of dysplastic cells, blasts, and young forms of the same cell line is also important. Masses of apparently proliferating cells are best found in preparations made from selected marrow bits. When the percentage of characteristic cells is low, consideration of the clinical

TABLE 2.—*Results of Treatment with Stilbamidine*

Case no	Stilbamidine treatment	Total dose grams	Diet	Basophilic inclusions in cells	Relief of pain	General effect	Final results
1 A S	1/31 to 2/18/47	2.7	Normal	No	No	None	Died
2. R L	2/6 to 2/26/47	2.8	Normal	Yes	Yes	Improved	Poor Increase in myeloma cells and osteolytic lesions
	4/15 to 4/25/47	1.3	Low animal protein	Yes	Yes	Neuropathy of 5th nerve	Persistent burning of face
3 C H	3/3 to 3/22/47	2.85	Low animal protein	Yes	Yes	Transient improvement	Poor
4 A W	7/18 to 8/9/47	2.85	Diabetic protein 97 grams	No	No	None	
	8/18 to 8/27/47	1.5	Low animal protein	No	No	None	Died
6 E. B	11/27 to 12/12/47	1.65	Low animal protein	No	No	None	Died

picture as a whole is felt to be essential to the diagnosis. This, of course, is always preferred, so that marrow puncture becomes only one of the criteria upon which diagnosis is based.

The thesis that multiple myeloma tissue is derived from a dysplastic line of plasma cells originating in the bone marrow is supported by our studies. All cases in this series are of the plasma cell type and plasmablasts, immature plasma cells, and dysplastic cells are described. A series of photo-micrographs (figs 4 to 15) offers objective evidence tending to confirm this theory.

Our observations confirm the original findings of Snapper that large basophilic inclusion bodies may be demonstrated in the cytoplasm of myeloma cells obtained

\* One patient with anemia due to chronic uremia revealed 10 per cent plasma cells in smears made from marrow bits when only an occasional plasma cell was seen in direct smears.



from bone marrow aspiration, in patients with multiple myeloma on a low animal protein diet, following treatment with stilbamidine. These basophilic bodies were not present prior to treatment. The two patients in our series who showed the granules obtained relief of pain. One patient was on a low animal protein diet while the diet of the second was not restricted. The latter patient showed typical basophilic granulation and a reduction in the percentage of plasma cells in marrow aspiration smears, and at the same time had a definite enlargement of osteolytic lesions in his skull. This fails to confirm the observation of Snapper that a low animal protein diet is essential for the production of basophilic granulation in the myeloma cells. The supposition that relief of pain is produced by an arrest of myeloma cell proliferation is also not substantiated. Complete failures to relieve pain or produce basophilic granules in the myeloma cells were recorded in three of our series of patients who had hyperglobulinemia. One was on a nonrestricted diet, and one received two courses of stilbamidine, the first without and the second with a low animal diet. The third patient was on a low animal protein diet.

Reactions to treatment with stilbamidine were transient except in one patient (case 3). He developed a trigeminal neuropathy which was still causing a severe burning sensation in his face after six months of observation. No dissociation of sensation occurred. Snapper reported an incidence of 10 cases of trigeminal neuropathy in a total of 18 patients treated with stilbamidine, and explained the mechanism as due to toxic degeneration of the principle sensory nucleus of the trigeminal nerve.<sup>13</sup> This caused severe and persistent itching which was disabling in character in only one of his patients. This subjective symptom ultimately disappeared in all of his patients. The objective findings of dissociated anesthesia were persistent.

#### SUMMARY

1. The value of bone marrow aspiration in the diagnosis of multiple myeloma was confirmed and discussed. This procedure should be utilized in all patients suspected of having this disease.

2. The importance of a reliable technic of studying bone marrow obtained by aspiration was stressed, and a method emphasizing certain important features described in detail.

3. The theory that multiple myeloma is derived from a dysplastic line of plasma cells originating in the bone marrow was supported by this study.

4. The original observation of Snapper has been confirmed, that after treatment of multiple myeloma patients with stilbamidine, large basophilic inclusion bodies can be demonstrated in the cytoplasm of a majority of myeloma cells obtained from bone marrow aspiration and stained by Wright's stain. This was produced on a nonrestricted as well as on a low animal protein diet.

5. Relief of pain was produced in two out of five patients with multiple myeloma treated with stilbamidine. One patient who was relieved of pain was on a low animal protein diet while the diet of the second was unrestricted. In both cases basophilic inclusion bodies appeared in the myeloma cells following treatment. Stilbamidine therapy failed to alleviate pain or to produce basophilic granulation in the myeloma cells in three patients who exhibited hyperglobulinemia.

- 6 Relief of pain and vertigo occurred in one patient treated with stilbamidine while osteolytic lesions were observed to enlarge by roentgenological examination
- 7 Trigeminal neuropathy with severe discomfort still continued six months following treatment in one patient
- 8 An arrest or remission in the course of the disease was not obtained in five cases of multiple myeloma treated with stilbamidine

## REFERENCES

- <sup>1</sup> JONES H B On a new substance occurring in the urine of a patient with mollities ossium *Phil Tr* London 138 55-62 1848
- <sup>2</sup> VON RUSTIZKY, J Multiples myelom *Deutsche Ztschr f Chir* 3 162-172, 1873
- <sup>3</sup> KÄHLER O Zur Symptomatologie des Multiplen Myeloms Beobachtung von Albumosurie *Prag Med Wehnschr* 14 33-45 1889
- <sup>4</sup> ATAINSON F R B Multiple myelomata *M Press* 195 312, 327 1937
- <sup>5</sup> BAYRD E D AND HECK F J Multiple myeloma a review of eighty three proved cases *J A M A* 133 147-157 1947
- <sup>6</sup> DIOGE L W AND SIRRIDGE M S A study of the sternal marrow and peripheral blood of fifty five patients with plasma cell myeloma *J Lab & Clin Med* 32 167-177, 1947
- <sup>7</sup> SNAPPER I On the influence of stilbamidine upon multiple myeloma *J Mt Sinai Hosp* 13 119-127, 1946
- <sup>8</sup> SNAPPER I, AND SCHNEID B On the influence of stilbamidine upon myeloma cells *Blood* 1 534-536 1946
- <sup>9</sup> KOPAC M I Cellular mechanisms in chemotherapy *Tr N Y Acad Sc* 8 5-10 Series 2, 1945
- <sup>10</sup> SNAPPER, I AND SCHNEID B The development of basophilic inclusion bodies in myeloma cells after stilbamidine treatment, *Ann Int Med* 27 541-547 1947
- <sup>11</sup> — MIRSKY A E RIS H SCHNEID, B AND ROSENTHAL, M Development of inclusion bodies containing ribose nucleic acid in myeloma cells after injections of stilbamidine Determination of stilbamidine in myeloma tissue *Blood* 2 311-322, 1947
- <sup>12</sup> ARAI H AND SNAPPER I The influence of stilbamidine upon kidney function, liver function and peripheral blood in multiple myeloma *Neurologic Sequelae of Stilbamidine Therapy N Y S J Med* 47 1867-1874 1947
- <sup>13</sup> SNAPPER I Stilbamidine and Pentamidine in Multiple Myeloma *J A M A* 133 157-161, 1947
- <sup>14</sup> PROPP S AND SCHWIND, J L Sternal puncture as a practical diagnostic procedure *Ann Int Med* 21 580-606, 1944
- <sup>15</sup> DOAN C A Bone marrow *Handbook of Hematology* (Downey H) New York, Paul B Hoeber Inc 1938
- <sup>16</sup> BLOOM W Lymphatic tissue Lymphatic organs *Handbook of Hematology* (Downey, H) New York Paul B Hoeber, Inc 1938
- <sup>17</sup> ROSENTHAL, N AND VOORL P Value of sternal puncture in diagnosis of multiple myeloma *J Mt Sinai Hosp* 4 1001-1019 1938

# THE DISTRIBUTION OF HISTOCHEMICALLY DEMONSTRABLE GLYCOGEN IN HUMAN BLOOD AND BONE MARROW CELLS

By MAX WACHSTEIN, M D

RENEWED attention has recently been given to the glycogen content of the human blood Wagner,<sup>17</sup> confirming older observations in the literature,<sup>1 18</sup> found glycogen only in the granulated leukocytes by chemical estimation, while the other formed elements in normal blood did not contain this substance Increased amounts of glycogen were found in the blood of patients with myeloid leukemia, while in the blood of patients suffering from acute leukemia, as well as of those with chronic lymphatic leukemia, normal amounts of glycogen were present <sup>1 18</sup>

The particular usefulness of the periodic acid-Feulgen technic for the histochemical demonstration of polysaccharides (McManus,<sup>11</sup> Lillie,<sup>10</sup> Hotchkiss<sup>9</sup>), prompted an examination of human blood and bone marrow smears

## TECHNIC

Either air-dried blood and bone marrow smears, or films fixed in absolute alcohol were placed into a 0.5 per cent solution of periodic acid in water for five minutes Even slides several years old gave very satisfactory staining reactions After washing in tap water, the slides were immersed in Schiff's reagent, prepared according to Hotchkiss,<sup>9</sup> for fifteen minutes They were then rinsed in three changes of SO<sub>2</sub> containing water, each time for two minutes, and then washed for five minutes under tap water Harris hematoxylin was used as counterstain After the usual dehydration, slides were covered with a resin under cover slips In order to identify the stained substance as glycogen, alcohol-fixed blood and bone marrow films were first covered with saliva for 15-30 minutes at room temperature <sup>2</sup> After a short wash in distilled water, the above described staining technic was employed

## RESULTS

*Normal blood smears* Only the polymorphonuclear leukocytes and platelets showed consistent staining The cytoplasm of the polymorphonuclear leukocytes revealed a dark red to Bordeaux red uniform color (fig. 1) Occasionally it contained small dustlike and also somewhat coarse granules The nucleus was always stained At least 90 per cent of all polymorphonuclear leukocytes gave this staining reaction Eosinophiles in normal blood, as well as in films in which markedly enlarged amounts of these cells were present, showed faint staining of their cytoplasm The granules remained unstained Lymphocytes were mostly without staining However, some showed small granules in the cytoplasm Monocytes were either negative or showed only faint staining The thrombocytes consistently gave a positive reaction For the most part they showed a brilliantly stained center and a paler outer border

From the Laboratories of the St. Catherine's Hospital Brooklyn New York and the Division of Pathology, Mt. Sinai Hospital, New York New York

*Leukocytosis* In blood smears from patients suffering from marked infectious leukocytosis due to various causes, polymorphonuclear leukocytes, including the metamyelocytes, contained large amounts of cytoplasmic glycogen. The cytoplasm showed not only diffuse staining, but in some cells it appeared in coarser granules than commonly seen in the blood films of normal subjects.

*Infectious mononucleosis* The atypical lymphocytes were negative with the exception of some which contained dark red granules as seen in normal lymphocytes.

*Various anemias* In smears from patients with various anemias (erythroblastosis fetalis, Cooley's anemia, etc.) nucleated red cells, erythroblasts, as well as normoblasts, were negative.



FIG. 1. A blood film from a normal individual stained by the periodic acid-Schiff technic for glycogen. Two polymorphonuclear leukocytes show diffuse staining of the cytoplasm. The nuclei are counterstained with Harris hematoxylin.

*Lymphatic leukemia* The lymphocytes found in blood smears of patients suffering from lymphatic leukemia showed the same staining reaction as those in normal blood smears.

*Chronic myeloid leukemia* Myelocytes, metamyelocytes, and polymorphonuclear leukocytes showed the cytoplasmic polysaccharide reaction. Myelocytes took only a faint reddish color (fig. 2). The intensity of the reaction obviously increased with the maturation of the cells. Dark red granules and coarse stippling were fairly frequent in polymorphonuclear leukocytes.

*Acute myeloid leukemia* Most of the myeloblasts did not contain glycogen, while some cells, obviously still quite immature, revealed occasional dark red granules or even a brim of red-staining cytoplasm around the large immature nucleus. More mature myeloid elements showed the usual amount of glycogen.

*Blood smears from various animals* The polymorphonuclear (heterophile) leukocytes in the blood film of dogs, rabbits, guinea pigs and frogs showed considerable staining reaction. Only faint traces were demonstrable in the white cells of the rat and mouse.

*Smears from Lymph nodes* In films from tonsils and lymph nodes not involved by disease, the lymphocytes showed no trace of glycogen.

*Bone marrow* In films from bone marrow of normal individuals, as well as of those with various abnormal conditions, a behavior of the myeloid elements similar to that seen in the blood films of patients with chronic myeloid leukemia was observed. Myeloblasts were mostly negative. As the myeloid cells matured, the stain



FIG. 2. A blood film from a patient with chronic myeloid leukemia, stained by the periodic acid Schiff technic for glycogen. Three polymorphonuclear leukocytes—one juvenile cell and two myelocytes—are shown. The cytoplasmic staining is most pronounced in the mature cells.

ing reaction in the cytoplasm became more pronounced. Occasional myeloid cells showed coarse granules. Most nucleated red cells, including the typical megakaryoblasts of pernicious anemia, showed no staining reaction. A very occasional erythroblast revealed a faintly stained cytoplasm. Plasma cells as well as the atypical cells found in multiple myeloma, were mostly negative. A considerable proportion of the megakaryocytes gave a distinct reaction. The cytoplasm was coarsely stained. Only occasional megakaryocytes did not contain any stainable polysaccharides.

#### COMMENT

As far back as 1877, Ranvier<sup>12</sup> demonstrated glycogen in the leukocytes of the frog with the help of iodine. Ehrlich,<sup>6</sup> several years later, was the first to examine

films of human blood for its glycogen content. Since then a good number of papers dealing with the histochemical demonstration of glycogen have been published. The older literature has been extensively reviewed in Neukirch's<sup>12</sup> and Girardin's<sup>8</sup> contributions. So far, the following methods have been used for the demonstration of glycogen in blood cells:

1. *Iodine reaction*

(a) Dried films were exposed to iodine vapors (Ehrlich and Lazarus<sup>7</sup>). In normal blood films leukocytes are unstained, red blood cells take a brownish hue and platelets are stained. The leukocytes in exudates, however, show a strong reaction.

(b) Wet films were exposed to iodine vapors according to Zollikofer.<sup>22</sup> All neutrophilic leukocytes stain diffusely while some (about 20 per cent according to Girardin<sup>8</sup>) contain glycogen granules. As with Ehrlich's method the platelets are distinctly and the red blood cells faintly stained.

2. *Best's carmine method*

This method was modified by Neukirch<sup>12</sup> for blood films. All polymorphonuclear leukocytes in normal blood give a diffuse to fine granular staining. In addition the centers of the thrombocytes are stained. The other cells are unstained with the exception of occasional lymphocytes showing a few red granules. Neukirch found the eosinophilic granules positive in blood films and Arnold<sup>1</sup> in bone marrow section, while Girardin, using Neukirch's method, found them consistently negative.

3. *The Bauer-Feulgen stain* as well as a *silver technic*, have been used for bone marrow section of the normal rhesus monkey by Wislocki and Dempsey.<sup>21</sup> Glycogen was demonstrable in polymorphonuclear neutrophils and neutrophilic metamyelocytes but not in any other cells. In the circulating blood, as observed in sections of blood contained in the heart, glycogen was seen only in polymorphonuclear leukocytes.

As is well known, substances giving any of the reactions described above, can only be considered as glycogen if they can be digested by amylase. It has been repeatedly demonstrated that the carbohydrate-like substance found in the cells of purulent exudates can be digested by saliva.<sup>12</sup> According to Neukirch, however, with both the iodine or Best's carmine technic, the staining reaction in the leukocytes of the blood, as well as that in the platelets, is not prevented by previous treatment with saliva. Dempsey and Wislocki, on the other hand, using the Bauer-Feulgen technic, found the stainable substance in leukocytes digestible by saliva.

Further examination of the nature of the substance giving the reaction with the periodic acid-Schiff technic was therefore undertaken. By using periodic acid, polysaccharides are oxidized to polyaldehydes. The aldehyde group reacts with Schiff's reagent. Low molecular compounds such as simple sugars and hydroxyamino acids can also react with this reagent, while the pentose component of nucleic acid does not react (Hotchkiss).

No staining reaction was seen in air-dried or alcohol-fixed films without previous treatment with periodic acid, thus excluding the possibility that the reaction was due to preformed aldehyde groups. Since the reaction occurred after twenty-four hours of alcohol fixation it could not have been caused by the alcohol-soluble plasma. The substance was still present at room temperature (24-26°C) after immersion of the films up to 150 minutes in distilled water or saline solution. Therefore it appears unlikely that the reaction was due to the presence of simple low molecular water-soluble substances.

In order to prove that the stainable substance was really glycogen, blood films were subjected to digestion with saliva. A significant difference became apparent

when films were only air-dried or had been fixed with absolute alcohol. After fixation the stainable substance, in leukocytes as well as in platelets, disappeared fifteen to thirty minutes following salivary digestion at room temperature. In unfixed films, the diastatic effect of saliva was considerably less pronounced, although varying in intensity in different slides. Salivary digestion occurred to a varying degree in alcohol-fixed films from bone marrows as well as in the peripheral blood of patients with myeloid leukemia. In occasional films, the cells proved resistant to the diastatic enzyme.

The results of these experiments make it seem very probable that the substance giving the aldehyde reaction after treatment with periodic acid in blood cells is glycogen. The glycogen in hematic elements, however, is relatively resistant to salivary digestion, unless first treated with alcohol. This may be due to the fact that the cells contain the glycogen in some chemical combination, possibly with protein. Such an assumption was made many years ago by Best.<sup>3</sup> Willstaetter and Rhodewald<sup>19</sup> discuss the peculiar fact that the glycogen becomes more demonstrable in leukocytes of exudates than in the blood. Ehrlich thought that the leukocytes which migrate from the blood stream are being changed in such a way that after some time free glycogen occurs. According to Willstaetter and Rhodewald, it can be assumed that the glycogen is not present in its usual form but possibly in some absorption, or more probably, in chemical connection with the cell protein.

As has been previously found, employing iodine as well as Best's carmine, the platelets give a strong staining reaction. Moreover, in the bone marrow films most of the megakaryocytes are stained. This is obviously due to glycogen, since the staining reaction is prevented by digestion with saliva. In contrast, Wagner<sup>17</sup> found that the reducing substance which is formed after acid digestion of platelets was not digestible by yeast. He therefore concluded that it originated from ribonucleic acid rather than glycogen. By histochemical methods Wislocki, Bunting and Dempsey,<sup>21</sup> found some ribonucleoprotein in the cytoplasm of megakaryocytes.

The behavior of histochemically demonstrable glycogen resembles that of stainable oxydase. This, however, should not be expected to be of practical value for the differentiation of myeloid from lymphatic cells, since some myeloblasts as well as occasional lymphocytes reveal glycogen granules.

A certain parallel of the glycogen reaction in the leukocytes and the histochemically demonstrable phosphatase is quite obvious. Phosphatase activity becomes apparent in myelocytes.<sup>16</sup> The intensity of the phosphatase reaction increases with the maturation of the myeloid cell and is particularly prominent in films of patients with infectious diseases and in exudates. It was previously assumed that this increase in phosphatase may indicate an intensification of metabolic processes.<sup>16</sup> Glycogen is probably the main source of energy for the leukocytes.<sup>18, 22</sup> The importance of phosphate-splitting enzymes in the intermediary carbohydrate metabolism is well recognized. The histochemically demonstrable relationship between glycogen and phosphatase activity has recently been discussed by Dempsey and Wislocki.<sup>5</sup>

#### SUMMARY

By applying Schiff's reagent after periodic acid treatment to blood and bone marrow films, a cytoplasmic staining reaction is seen in some cells of the myeloid

series, as well as in megakaryocytes and platelets. The intensity of the staining reaction in the myeloid cells increases with their maturation. The staining reaction can be prevented altogether in alcohol-fixed films by salivary digestion, but only incompletely in air-dried films. The staining reaction is due to the presence of glycogen in some chemical association, possibly with protein.

## REFERENCES

- <sup>1</sup> ARNOLD J. Enthalten die Zellen des Knochenmarkes, die Eosinophilen insbesondere, Glycogen? *Centr allg Path u path Anat* 22 1-9, 1910
- <sup>2</sup> BENLEY, C. M. Comparison of methods for demonstrating Glycogen microscopically. *Stain Technol* 14 47-52, 1939
- <sup>3</sup> BEST. Ueber Glycogen, insbesondere seine Bedeutung bei Entzündung und Eiterung. *Beitr z path Anat u z allg Path* 33 584-604 1903
- <sup>4</sup> DE HAAN J. Ueber den Glycogengehalt der weissen Blutkörperchen. *Biochem Ztschr* 128 124-143, 1921
- <sup>5</sup> DEMPSEY E. W., AND WISLOCKI G. B. Histochemical contributions to physiology. *Physiol Rev* 26 1-28 1946
- <sup>6</sup> EHRLICH, P. Ueber das Vorkommen von Glycogen im diabetischen und im normalen Organismus. *Ztschr klin Med* 6 33-46 1883
- <sup>7</sup> EHRLICH, P. AND LAZARUS A. Die Anaemien, in Nothnagel. *Spezielle Pathologie und Therapie* ed 2, vol 8. Wien and Leipzig A. Hoeslger 1909
- <sup>8</sup> GIRARDIN, A. Zur Frage der jodophilen Substanz der Leukozyten. *Jahrb Kinderheilk* 145 140-166 1935
- <sup>9</sup> HOTCHKISS R. D. A microchemical reaction resulting in the staining of polysaccharide structures in fixed tissue preparations. *Arch Biochem* 16 131-141 1948
- <sup>10</sup> LILLIE, R. D., with the assistance of LASKER A. GRECO, J. AND JACQUIER H. Studies on the preservation and histologic demonstration of glycogen. *Bull Internat Assn Med Museum* 27 23-61 1947
- <sup>11</sup> McMANUS, J. F. A. The histological demonstration of mucin after periodic acid. *Nature* 158 202, 1946
- <sup>12</sup> NEUKIRCH, P. Ueber die jodophile Substanz der Leukozyten und ihr Verhalten zur Best. schen Färbung. *Ztschr klin Med* 70 251-256, 1910
- <sup>13</sup> RANVIER, L. *Anatomie generale*. Paris. *Progres Medical* 1877
- <sup>14</sup> SUTER E. Ueber den Glycogengehalt des Blutes unter verschiedenen physiologischen, pharmakologischen und pathologischen Bedingungen. *Helvetica physiol et pharmac acta* 5 6-22, 1947
- <sup>15</sup> UNTHHELM, E. Ueber das Glycogen im Blute des Menschen. *Z ges exp Med* 96 129-142, 1935
- <sup>16</sup> WACHSTEIN M. Alkaline Phosphatase activity in normal and abnormal human blood and bone marrow cells. *J Lab & Clin Med* 31 1-17 1946
- <sup>17</sup> WAONER R. The estimation of glycogen in whole blood and white blood cells. *Arch Biochem* 11 249-258, 1946
- <sup>18</sup> WAONER, R. Studies on the physiology of the white blood cell. The glycogen content of leucocytes in leukemia and polycythemia. *Blood* 2 235-243 1947
- <sup>19</sup> WILLSTÄTTER R., AND RHODEWALD M. Ueber die Amylasen der Leukozyten. *Ztschr phys Chem* 203 189-240, 1931
- <sup>20</sup> WILLSTÄTTER R., AND RHODEWALD, M. Ueber Aufbau und Abbau des Glycogenes durch Leukozyten (zehnte Abhandlung ueber Enzyme der Leukozyten). *Ztschr physiol Chem* 247 115-126 1937
- <sup>21</sup> WISLOCKI G. B. BUNTING H. AND DEMPSEY E. W. Further observations on the chemical cytology of megakaryocytes and other cells of hemopoietic tissues. *Anat Rec* 88 527-537 1947
- <sup>22</sup> ZOLLIKOFFER. Zur Jodreaktion der Leukozyten. *Bern Diss* 1899



# PYRIDOXINE DEFICIENT DIET AND DESOXYPYRIDOXINE IN THE THERAPY OF LYMPHOSARCOMA AND ACUTE LEUKEMIA IN MAN

By ALFRED GELFHORN, M D , AND LOGAN O JONES, M D

EVIDENCE from animal experimentation indicates that pyridoxine, a component of the vitamin B complex, is an essential factor for the maintenance and function of hematopoietic tissue. Severe anemia has been induced in dogs<sup>1</sup> and swine<sup>2</sup> on a pyridoxine deficient diet and the integrity of lymphoid tissue in rats has also been demonstrated to be dependent upon this vitamin.<sup>3-5</sup> The significance of pyridoxine in human nutrition is unknown inasmuch as no known deficiency state involving this vitamin alone has been described.<sup>6</sup> Recently, Stoerk reported that lymphosarcoma transplants failed to grow in mice maintained on a pyridoxine deficient diet, he further noted that established transplants of lymphosarcoma regressed when mice were placed on a pyridoxine deficient diet together with the specific vitamin antagonist, desoxypyridoxine.<sup>7</sup>

In the experiments reported in this paper, an attempt was made to induce a pyridoxine deficiency in man to determine any possible therapeutic effects in lymphosarcoma and acute leukemia. Although no significant clinical improvement resulted from the experimental therapeutic regimen, the results are of interest in that they indicate certain fundamental differences in the utilization of pyridoxine in lower animals and man.

## MATERIALS AND METHODS

Six patients were placed on a pyridoxine deficient diet and given desoxypyridoxine. Three patients had disseminated lymphosarcoma and 3 had acute leukemia. It was planned to keep the subjects on this diet for fourteen days. However, in several instances the caloric intake was limited so severely by the unpalatability of the diet that the regimen was of necessity discontinued earlier. The basic constituents of the diet were (a) vitamin-free casein,\* (b) gelatin, (c) sugar, (d) cornstarch, (e) unenriched cream of wheat, (f) butter, (g) artificial flavoring. The patients were also allowed carbonated drinks such as ginger ale and Coca-cola ad libitum, tea and coffee without cream or milk, and one serving of peaches or one apple per day. The dietician, working with this very limited number of foodstuffs, prepared cookies and puddings to provide some variety. However, all of the food had a chalky consistency and flavor which rapidly became extremely distasteful to the patients. Therefore, although an adequate amount of protein, carbohydrate and fat, as well as an adequate number of calories were provided, in only 2 of the patients was the diet completely consumed. In addition to the above, the

From the Departments of Pharmacology and Medicine, Columbia University College of Physicians and Surgeons and the Presbyterian Hospital, New York.

This work was supported by a grant from the American Cancer Society on recommendation of the Committee on Growth of the Vocational Research Council.

\* Labco Borden & New York

patients were given appropriate doses of thiamine, nicotinic acid, riboflavin and ascorbic acid as individual vitamins

Desoxypyridoxine\* was given to the first 2 patients studied in doses of 25 mg per Kg per day in three equally divided doses by mouth. Due to toxic manifestations which will be described later, the dose was progressively decreased to approximately 2.5 mg per Kg per day in the other patients of the series.

Disturbance of tryptophane metabolism as manifested by xanthurenic acid and kynurenine excretion in the urine has been reported in experimental animals on a pyridoxine deficient diet.<sup>8-10</sup> Daily 24 hour urine specimens were collected on 2 of the patients and xanthurenic acid determinations were made according to the technique described by Porter.<sup>9</sup>

### CASE REPORTS

**Case 1 D T** This 6 year old white boy had developed painless swelling at the base of his neck with associated fever, cough and wheezing respirations beginning four weeks before hospital entry. Aside from recurrent asthma and eczema his past history was negative. Examination revealed slight fever (100.2 F) wheezing but unlabored respirations hoarse cough generalized lymphadenopathy massive in left cervical and axillary regions and probably retroperitoneal as well with splenomegaly. Positive x ray findings were limited to the chest where a widened mediastinum and a large anterior mediastinal mass were noted. Blood studies revealed normochromic anemia a normal leukocyte count and differential and normal sternal bone marrow. Cervical node biopsy disclosed *lymphocytic lymphosarcoma*.

Following a four day course of methyl bis ( $\beta$ -chloroethyl) amine totalling 0.4 mg per Kg the temperature fell to normal appetite and weight increased hoarse cough disappeared and visible nodes regressed. Within the ensuing week lymphadenopathy recurred. Shortly thereafter the patient was placed on a pyridoxine free diet for four days together with the pyridoxine analogue desoxypyridoxine (2 mg per Kg per day) and 1.0 Gm l tryptophane — both in three equal daily doses. L tryptophane was given to accentuate any possible disturbance in the metabolism of this amino acid. Throughout this period appetite decreased markedly, concomitant weight loss of 0.5 Kg and continued enlargement of nodes with tracheal compression were noted. Of interest was the fact that the peripheral blood values remained unchanged and no xanthurenic acid was excreted in any of the four 24 hour urine specimens during these four days. Lymph node response to a second course of methyl bis ( $\beta$ -chloroethyl) amine administered at this time was minimal and the patient was discharged to receive radiotherapy at an institution nearer his home after a lapse of two weeks. Although therapeutic response to irradiation appeared to have been excellent one month later he developed recurrent epistaxis rectal bleeding and abdominal pain and died at home. The total duration of his illness had been approximately four months.

**Case 2 R C** This 52 year old white housewife was hospitalized for progressive painless enlargement of all superficial lymph nodes over a six months period with increasing nasal obstruction and hearing loss as well as pain in the right hip. Pertinent physical findings were some loss of hearing hyperplasia of pharyngeal lymphoid tissue in addition to generalized lymphadenopathy (including retroperitoneal area probably) hepatosplenomegaly and fever. Blood corpuscle counts and differential urinalysis liver function tests and x rays of chest and sinuses were normal. Fasting serum sugar was 58 mg per cent. An inguinal node biopsy revealed *reticulum-cell sarcoma*.

The patient received a single injection of 36.4 mg (0.44 mg per Kg) of methyl bis ( $\beta$ -chloroethyl) amine through technical error the only objective toxic effects of which were marked nausea and vomiting minimal diarrhea without occult blood in stools and profound leukopenia. Because of poor and transient therapeutic response she was placed on a pyridoxine free diet with added desoxypyridoxine at three hour intervals during the day totalling 25 mg per Kg daily. After a total of 1.5 Gm of desoxypyridoxine had been given she developed persistent nausea and suddenly lost consciousness became cyanotic and exhibited mass movements of extremities of a convulsive character. Following spontaneous

\* Generously supplied by Dr. D. F. Robertson, Merck Institute for Therapeutic Research.

recovery in about two minutes 100 mg of pyridoxine was given parenterally. The following day residual sequelae were noted in the form of diaphoresis, nausea and some amnesia for recent events, and the dose of desoxypyridoxine was lowered to 2.5 mg per Kg per day in three equal doses. During a fourteen day period on the deficient diet, weight loss, mental confusion and a 50 per cent reduction in lymph node size occurred. Repeated blood counts showed no abnormalities or changes, and a fasting blood sugar of 51 mg per cent did not differ significantly from the control value. On the hypothesis that additive effects might be obtained, a four day course of methyl bis ( $\beta$ -chloroethyl) amine was then given, totalling 0.4 mg per kg, there was further regression of adenopathy and some weight gain. The patient died suddenly within two weeks following hospital discharge at another institution. No necropsy was performed. The total duration of her illness had been nine months.

**Case 3 J S** This 52 year old white salesman entered the hospital with painless enlargement of the glands in his neck over a seven week period associated latterly with dysphagia, weakness and weight loss. Examination disclosed generalized lymphadenopathy particularly in the neck, hepatosplenomegaly and signs of fluid at the right lung base. Peripheral blood showed no anemia but a leukocytosis with a relative increase in lymphocytes some of which were immature forms. X rays of the chest revealed a right paratracheal shadow and confirmed the clinical impression of right pleural effusion. Biopsy of an axillary node disclosed *reticulum-cell lymphosarcoma*. Therapeutic response to a four day course of methyl bis ( $\beta$ -chloroethyl) amine totalling 0.4 mg per Kg was poor so that adenopathy remained stationary, right pleural fluid increased in amount and an exhausting nonproductive cough developed. Chest fluid revealed histologic changes compatible with lymphosarcoma at this time. The patient was then placed on a pyridoxine deficient diet with added desoxypyridoxine totalling 2.5 mg per Kg daily in three divided doses. During this period anorexia and cachexia became marked and he exhibited mental dullness, somnolence, increasing cough and edema of both legs and low grade afternoon fever. Some observers felt cervical lymph nodes became softer and smaller during this two weeks. The blood picture remained unchanged throughout and no xanthurenic acid excretion was detected in 24 hour urine specimens. Forty eight hours after resuming a regular diet the patient died suddenly. His illness had lasted about three months. Autopsy performed 100 hours postmortem showed gross evidence of widely disseminated invasive lymphosarcoma involving liver, spleen, heart, lungs, stomach and kidney as well as nodes in the mediastinum and abdomen. Microscopic examination confirmed these findings and in addition showed similar changes in prostate, bone marrow and thyroid gland. The nervous system appeared grossly and microscopically normal. Splenic sections were of interest in that scattered fields showed multinucleated lymphoid cells, fragmentation of nuclear material and minimal necrosis about lymphoid cell clusters. Dr. H. C. Stoerk who kindly reviewed these sections stated these latter sections bore some resemblance to changes seen in lymphoid tumors of pyridoxine deficient animals. However, in the main, comparison of the morphology of the tumor from autopsy material showed no significant variations from that seen in the pretreatment biopsy sections.

**Case 4 G deC** This 8½ year old white boy had a 3 week history of pharyngitis, cervical adenopathy and fever and showed hypertrophic gums, enlargement of the liver and all superficial nodes. Blood studies including sternal marrow aspiration were compatible with the diagnosis of *acute monocytic leukemia*. After fourteen days on a pyridoxine free diet with added vitamin antagonist, physical and hematologic findings remained unchanged. During the ensuing month his condition degenerated rapidly with continued fever, weight loss and hemorrhagic phenomena and he died at home in the ninth week of his illness.

**Case 5 C U** Blood studies confirmed the clinical impression of *acute lymphatic leukemia* in this 3 year old white male with symptoms of one month duration and fever, extensive purpura and marked hepatomegaly on physical examination. During 13 days of pyridoxine deficient diet with added antagonist the WBC dropped from 6100 to 1650, blast forms from 70 to 25 per cent and RBC from 2.39 to 1.54 millions with parallel hemoglobin changes. Ten days later the patient died in coma. No autopsy was performed. Total duration of his disease was two months.

**Case 6 J K** This 4 year old white male with symptoms of six weeks duration showed pallor, generalized adenopathy, hepatosplenomegaly and petechiae. Blood studies showed a high percentage of blasts and a diagnosis of *acute leukemia* was made. On the fifth day of a pyridoxine deficient diet with added desoxypyridoxine totalling 25 mg per kg he had two generalized convulsive seizures in rapid succession following which the desoxypyridoxine was reduced to 2.5 mg per kg. Because the child be-

came irrational for several hours twenty four hours later, the latter medication was discontinued, but the dietary regimen was prolonged until death eight days after its institution. During this period a marked and progressive leukocytosis with an increase in blasts from 90 to 100 per cent and a fall in RBC and Hgb values were noted. Death occurred at the end of this period in coma after an illness totalling two months duration. No autopsy was performed.

### DISCUSSION

In 2 of the 3 patients with lymphosarcoma there was evidence of moderate regression in the size of the lymph nodes. It is unwarranted to ascribe this change specifically to a pyridoxine deficiency, for Stoerk has shown that in rats exposed to adverse dietary conditions there is an approximately linear relationship between the body weight deficit and the thymic weight deficit.<sup>4</sup> On the reasonable assumptions that (a) an analogous situation pertains to man and (b) that the decrease in the weight of the thymus is an expression of generalized lymphoid atrophy, it is probable that the observed changes in the lymphadenopathy of our patients were coincident with the nonspecific malnutrition. This conclusion is further strengthened by the fact that there were no demonstrable morphologic evidences of specific cellular change attributable to pyridoxine deficiency in the tumor of the patient who came to necropsy.

The marked depression of hematopoietic function described in pyridoxine deficient experimental animals was not clearly demonstrated in the patients of this study. Admittedly it is difficult to assess this particular point in patients with acute leukemia and widely disseminated lymphosarcoma. Since all of the cases had anemia of varying degrees of severity at the onset of the dietary regimen, it was impossible to determine the effect of the diet and desoxypyridoxine on erythropoiesis. It is to be noted, however, that leukopenia, lymphocytopenia, and thrombocytopenia did not occur during the period of observation except in one case of acute leukemia. In this patient there was no significant alteration of the hemogram and the development of leukopenia is entirely compatible with the natural course of the disease.

The two episodes of central nervous system excitation seen in our patients were probably an expression of acute pyridoxine deficiency induced by the large doses of the pyridoxine antagonist, desoxypyridoxine. Mushett et al.<sup>11</sup> have reported convulsions in experimental animals given large doses of desoxypyridoxine, and Wintrobe and his associates have described convulsive seizures in pyridoxine deficient swine which closely resemble grand mal epilepsy.<sup>12</sup> The signs and symptoms seen in our patients were also similar to this cerebral dysrhythmia.

Kynurenine and xanthurenic acid are abnormal metabolites of tryptophan which are excreted in the urine of animals which are deficient in pyridoxine.<sup>8-13</sup> Xanthurenic acid excretion in man has not been noted<sup>10</sup> and this has suggested that tryptophan degradation varies in different species. To our knowledge, such a rigorous pyridoxine deficient diet has not previously been employed in the studies of xanthurenic acid excretion in humans. Inasmuch as 2 of the patients failed to excrete xanthurenic acid while on the diet and while receiving desoxy pyridoxine, additional circumstantial evidence is provided that tryptophan is not metabolized

in the same way in all species, however, the possibility that the patients were not deficient in pyridoxine cannot be excluded

The criteria of a pyridoxine deficiency in experimental animals include depression of hemopoiesis, atrophy of lymphoid tissue, demyelinating lesions of the central and peripheral nervous system, and disturbance of tryptophane metabolism. Applying these criteria to the patients reported here would lead to the conclusion that (a) either a pyridoxine deficiency had not been induced or (b) pyridoxine is not essential in human nutrition. A final possibility is that the manifestations of vitamin B<sub>6</sub> deficiency in man are entirely different from those observed in animals and that they were unrecognized in the patients of this study. It is impossible to state with assurance which of the above possibilities pertained in these experiments. In mice, Stoerk has observed lymphoid regression within five days after the onset of desoxypyridoxine and a pyridoxine restricted diet.<sup>12</sup> This would indicate that there are not large stores of the vitamin in the body. Wintrobe and his associates, on the other hand, noted that in swine on a pyridoxine deficient diet two to seven weeks passed before there were unmistakable signs of the specific vitamin deficiency.<sup>13</sup> Since there was clear evidence of nonspecific malnutrition in our patients within fourteen days, a more protracted period of dietary experimentation was not justifiable.

It may be stated unequivocally that, under the conditions of the experiment, there was no evidence that restriction of the pyridoxine intake together with desoxypyridoxine had any therapeutic value. In the 3 patients with lymphosarcoma, a course of nitrogen mustard (methyl bis (beta chloroethyl) amine) was given following the completion of the experimental dietary period to determine whether a greater response would be produced by the chemotherapeutic agent. There was no significantly greater regression of the tumor masses observed following the post-pyridoxine deficiency nitrogen mustard therapy than had occurred with previous chemotherapy.

#### SUMMARY AND CONCLUSIONS

Three patients with disseminated lymphosarcoma and 3 cases of acute leukemia were placed on a pyridoxine deficient diet together with desoxypyridoxine for periods of four to fourteen days. Although there was evidence of malnutrition in the form of weight loss and weakness, none of the signs of specific pyridoxine deficiency described in animals was observed in the human. There was no unequivocal evidence of depression of hemopoiesis, no significant atrophy of lymphoid tissue, no signs of demyelination of nerves, and no abnormality of tryptophane metabolism determined by urinary xanthurenic acid excretion. The possibility that the dietary restriction of pyridoxine was too brief for the development of a deficiency syndrome was considered, but it was pointed out that the rigors of the procedure were too great to justify more protracted periods of observation.

Two patients had acute toxic manifestations following the administration of large doses of desoxypyridoxine. These were characterized by transient epileptiform convulsions. There were no sequelae and no recurrence of the symptoms when the dose of the drug was reduced.

There was no evidence that the restriction of pyridoxine in the diet together with desoxy pyridoxine for periods up to two weeks had any therapeutic effect in lymphosarcoma or acute leukemia. Also, no potentiation of the cytotoxic effect of nitrogen mustard was observed in the patients with lymphosarcoma when chemotherapy was given after the completion of the pyridoxine deficient regimen. It is to be emphasized that this does not absolutely exclude the possibility that pyridoxine deficiency may adversely affect lymphosarcoma in man. The short duration of the experiment and the well known refractoriness to any form of therapy of the tumors in these patients are factors which may have militated against a satisfactory outcome of the regimen.

## REFERENCES

- <sup>1</sup> McKIBBIN, J M, SHAEFER, A E, FROST D V AND ELVERJEM C A. Studies on anemia in dogs due to pyridoxine deficiency. *J Biol Chem* 142 77 1942.
- <sup>2</sup> CARTWRIGHT, G E, WINTROBE, M M, BUSCHKE W H, FOLLIS R H JR, SUKSTA A, AND HUMPHREYS S. Anemia, hypoproteinemia and cataracts in swine fed casein hydrolysates or zein. Comparison with pyridoxine deficiency anemia. *J Clin Investigation* 24 268 1945.
- <sup>3</sup> STOERK, H C AND ZUCKER T F. Nutritional effects on the development and atrophy of the thymus. *Proc Soc Exp Biol & Med* 56 151 1944.
- <sup>4</sup> ———. Effects of calcium deficiency and pyridoxine deficiency on thymic involution (accidental involution). *Proc Soc Exp Biol & Med* 62 90 1946.
- <sup>5</sup> ———. EISEN, H N AND JOHN H M. Impairment of antibody response in pyridoxine deficient rats. *J Exp Med* 85 365 1947.
- <sup>6</sup> CARTWRIGHT G. Dietary factors concerned in erythropoiesis. *Blood* 2 111 1947.
- <sup>7</sup> STOERK H C. The regression of lymphosarcoma implants in pyridoxine deficient mice. *J Biol Chem* 171 437 1947.
- <sup>8</sup> LEFKOVSKY S, ROBOZ, E AND HAAOEN-SMIT A J. Xanthurenic acid and its role in the tryptophane metabolism of pyridoxine deficient rats. *J Biol Chem* 149 195 1943.
- <sup>9</sup> PORTER, C C, CLARK I, AND SILBER R H. The effect of pyridoxine analogues on tryptophane metabolism in the rat. *J Biol Chem* 167 573 1947.
- <sup>10</sup> CARTWRIGHT, G E, WINTROBE M M, JONES P, LAURITSEN, M AND HUMPHREYS, S. Tryptophane derivatives in the urine of pyridoxine deficient swine. *Bull Johns Hopkins Hosp* 75 35 1944.
- <sup>11</sup> MURRETT, C W, STEBBINS, R B, AND BARTON M N. Histologic changes produced by 2,4-dimethyl 3 hydroxy 5 hydroxymethyl pyridoxine, an analogue of pyridoxine. *Am J M Sc* 213 509 1947.
- <sup>12</sup> WINTROBE, M M, FOLLIS R H JR, MILLER M H, STEIN H J, ALCTAOA R, HUMPHREYS, S, SUKSTA S AND CARTWRIGHT G E. Pyridoxine deficiency in swine with particular reference to anemia, epileptiform convulsions and fatty liver. *Bull Johns Hopkins Hosp* 72 1 1943.
- <sup>13</sup> STOERK, H C. Personal communication.

## A RAPID DIAGNOSTIC TEST FOR SICKLE CELL ANEMIA

By HARVEY A. ITANO, M.D., AND LINUS PAULING, Ph.D.

**S**ICKLE cell anemia is a congenital chronic hemolytic type of anemia characterized hematologically by the development of oat-shaped and sickle-shaped erythrocytes. Other cellular abnormalities which are due to excessive blood destruction and active blood formation are also seen in blood smears. Six to 10 per cent of Negroes possess the sickle trait<sup>2, 3</sup>, their red blood cells have the capacity to sickle, but most of these individuals do not develop anemia.

The course of the sickling process as observed under the microscope has been described in detail by several investigators,<sup>4, 5, 6, 10</sup> but little is known about the physical processes involved in sickling. It has been established, however, that the erythrocytes of individuals with sickle cell anemia and sickle cell trait become sickled when the hemoglobin is reduced.<sup>8, 14</sup> When the hemoglobin is combined with oxygen or carbon monoxide, the cells are indistinguishable in form from normal erythrocytes. The term promeniscocyte has been applied to the latter form and meniscocyte to the former.<sup>11</sup> Hahn and Gillespie<sup>8</sup> and Sherman<sup>14</sup> obtained sickling physically by reducing the partial pressure of oxygen over suspensions of promeniscocytes. They were able to reverse the process by passing oxygen or carbon monoxide over meniscocytes. When oxygen is removed from promeniscocytes, their hemoglobin aggregates in one or more foci within the cells, and the cell membrane collapses. When oxygen is added to these cells, they resume their normal contour, and hemoglobin appears to be distributed uniformly throughout their interior. Meniscocytes are strongly birefringent under the polarizing microscope<sup>14</sup> while promeniscocytes are not.

When a drop of blood is sealed between a cover slip and a slide, the decline in oxygen tension due to oxidative processes in the blood cells leads to sickling.<sup>7</sup> This is the common diagnostic test for sickle cell anemia and sickle cell trait used in clinical laboratories. Sherman found that increase in temperature, high leukocyte count, and bacterial contamination, all of which increase the rate of oxygen consumption, accelerated the sickling process. In another method, a saline citrate suspension of blood is allowed to stand in a test tube under a layer of paraffin oil until sickling takes place.<sup>1</sup> In employing any of the common diagnostic tests for sickling it is desirable to obtain blood which has a low fraction of oxyhemoglobin. Thus, the moist stasis method,<sup>13</sup> in which blood is obtained from a patient's finger after its circulation has been occluded for five minutes, gives the most rapid and consistent results. Even with this method it is sometimes necessary to observe the preparation for several hours before the result is conclusive.<sup>8</sup>

In order to find a more convenient and rapid method of producing meniscocytes

Contribution No. 1186 from the Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena, Calif.

This work was done under a grant from the U. S. Public Health Service and was briefly reported by Dr. Dan H. Campbell at the Hematology Symposium of the U. S. Public Health Service held at St. Louis, Mo., on February 2, 1948.

we turned to chemical reducing agents. Sodium dithionite,  $\text{Na}_2\text{S}_2\text{O}_4$ , rapidly reduces oxyhemoglobin to reduced hemoglobin, and this property suggested its use in testing erythrocytes for sickling. When a solution of sodium dithionite was added to promesococytes, nearly all of the cells showed sickling or the early changes in the sickling process within a few seconds. Dithionite ion tends to decompose to thiosulfate and sulfite with formation of hydrogen ion<sup>9</sup> so that solutions made up from commercial preparations of sodium dithionite are often strongly acid in reaction, but by adding  $\text{Na}_2\text{HPO}_4$  to the solutions it is possible to increase the pH and at the same time provide a buffering medium. Hahn and Gillespie found that sickling was obtained most consistently if cell suspensions were buffered at a slightly acid pH. We have prepared a satisfactory reagent by adding 0.114 M aqueous  $\text{Na}_2\text{HPO}_4$  to 0.114 M aqueous  $\text{Na}_2\text{S}_2\text{O}_4$  until the final pH was 6.8. The ratio of the volumes of  $\text{Na}_2\text{HPO}_4$  and  $\text{Na}_2\text{S}_2\text{O}_4$  necessary to obtain this pH was about three to two.

The blood used in the following experiments was obtained from 6 different cases of sickle cell anemia, 3 of whom were being treated for exacerbations and 3 of whom were in remission. An excess of the dithionite reagent was added to promesococytes on a microscope slide, almost immediately changes were evident in the erythrocytes. Typical crescentic forms did not appear in large numbers, presumably because of the time required for the reduced hemoglobin molecules to become oriented in what Ponder calls the paracrystalline state.<sup>11</sup> However, nearly all of the cells underwent changes in contour, and other changes described by earlier observers took place at an accelerated rate. The forms of many of these cells corresponded to the holly wreath cells of Sherman and cells classified as "abnormal" by Reinhard and his co-workers.<sup>12</sup> After about fifteen to thirty minutes the aggregates of hemoglobin in many of the cells became birefringent. The presence of so many holly wreath cells is in accord with Sherman's observation that this form appears in large numbers when the rate of removal of oxygen is rapid. Since dithionite does not react with carbon monoxide, promesococytes saturated with carbon monoxide would not be expected to undergo changes in contour upon addition of this reducing agent. This is indeed the case. Although no sickle cell trait blood was available to us for study, there is good reason to believe that such blood would behave in the same manner as sickle cell anemia blood.\*

#### METHOD

The rapidity and simplicity of this test suggests that it would be useful as a clinical laboratory procedure for diagnosing sickle cell anemia and sickle cell trait. No special precautions are necessary in collecting the blood for this test; oxygenated cells may be used since an excess of reducing agent can always be added. The test works equally well with oxalated blood or fingertip puncture specimens and may be applied in several ways. (1) About 0.05 ml. of reagent may be added to a very small drop (about 0.01 ml.) of blood on a slide. A cover slip is then laid over

\* A brief note by da Silva (Science 107: 221 (1948)) which appeared since the preparation of this paper indicates that he has successfully identified sickle cell trait by a procedure similar to method (1) below.



the mixture and cells observed under a microscope (2) An excess of reagent may be added to a small volume of blood in a test tube and a drop of the mixture observed (3) A convenient method for studying the entire process of sickling in a short period of time involves the use of a hemocytometer counting chamber The chamber is half filled with a dilute saline suspension of promiscocytes, the reagent is then added to fill the rest of the chamber The erythrocytes may be observed as the reducing agent diffuses into the part of the chamber which they occupy

Since the dithionite reagent is unstable as mentioned above, its reducing power should be tested frequently by the addition of a test portion to a dilute suspension of oxygenated erythrocytes If the reagent is satisfactory, a change from the color of oxyhemoglobin to that of reduced hemoglobin should be observed A large volume of stock  $\text{Na}_2\text{HPO}_4$  solution may be prepared, but it is desirable to make up the  $\text{Na}_2\text{S}_2\text{O}_4$  solution daily

#### ACKNOWLEDGMENTS

We are indebted to Dr Edward K Evans and D Travis Winsor for their aid in obtaining the blood used in these experiments

#### REFERENCES

- <sup>1</sup> BECK J S P, AND HERTZ C S *Am. J Clin Path* 5 315, 1935
- <sup>2</sup> CARDOZO W W *Arch Int Med* 60 623 1937
- <sup>3</sup> DIOGS, L W, AHMANN C F, AND BIBB, J *Ann Int Med* 7 769 1933
- <sup>4</sup> ——— AND BIBB J J *A M A* 112 695, 1939
- <sup>5</sup> ——— AND PETTIT, V D *J Lab & Clin Med* 25 1106, 1940
- <sup>6</sup> GRAHAM G S AND McCARTY, S H *J Lab & Clin Med* 12 536 1917
- <sup>7</sup> HAHN E V *Am J M Sc* 175 106 1918
- <sup>8</sup> ——— AND GILLESPIE E B *Arch Int Med* 39 233 1927
- <sup>9</sup> LATIMER W M *The Oxidation States of the Elements and their Potentials in Aqueous Solutions*  
Prentice Hall Inc, 1938
- <sup>10</sup> MURPHY R C AND SHAPIRO S *Arch Int Med* 74 28 1944
- <sup>11</sup> PONDER E *Ann N Y Acad Sc* 48 579 1947
- <sup>12</sup> REINHARD E H MOORE C V DUBACH R, AND WADE, L J *J Clin Investigation* 23 682, 1944
- <sup>13</sup> SCRIVER J B AND WAUGH T R *Canad M A J* 23 375 1930
- <sup>14</sup> SHERMAN I J *Bull Johns Hopkins Hosp* 67 309 1940

# THE MECHANISM OF PETECHIAL HEMORRHAGE FORMATION

By J G HUMBLE, M R C S , L R C P

THE OCCURRENCE of petechial hemorrhages in the skin and mucous membranes is a well known sign in many hemorrhagic diseases and in other complaints. Little is known, however, of the mechanism by which they are produced or the exact segment of the vessel or vessels at fault. Histologic preparations show merely an exudation of red cells, sometimes of polymorphonuclear neutrophil leukocytes around the minute vessels in the dermis, the vessel walls themselves usually appearing intact. In the present investigations the formation of petechiae by the tourniquet test (capillary resistance test) in the skin of patients suffering from various hemorrhagic diseases has been studied by capillary microscopy.

## METHOD AND APPARATUS

The patients lay comfortably in bed, the arm extended at right angles to the trunk, in the position of full supination resting on a firm table. The cuff of a blood pressure apparatus was adjusted to the upper arm and the skin in the antecubital fossa was shaved to remove hair and superficial squama. The area chosen was then covered with cedar wood oil. A Leitz Ultra-Pak microscope was used to study the skin vessels ( $11\times$  objective with the dipping cone attached and a  $10\times$  ocular). By adjustment it is possible to obtain optical continuity from the oil to the lens system to avoid surface glare from the oiled skin. A clear view of the blood in the minute skin vessels is thus obtained at a magnification of  $110\times$ . The vessels that are seen with the cuff uninflated are few and far between and are the terminal capillary loops. They are usually found in clusters of three or four. The cuff is then gently inflated to a pressure of 90-100 mm. of mercury (or between the systolic and diastolic blood pressure). As the venous system of the skin fills with blood it is possible to see the previously invisible superficial plexus of minute venules and the connections of the end capillary loops to this plexus. In the creases of the elbow it is also possible to see the end of the precapillary arteriole by the blood spurting into view as the capillary loop from the depths of the skin (fig. 1). It is at this point that petechial hemorrhages form, irrespective of the type of disease studied. The behavior of the exuded blood and the shape and character of the lesion formed is, however, different in the various types of disease studied. The following cases have been thus studied.

- (1) Essential Thrombocytopenic Purpura—5 cases  
Thrombocytopenic purpura secondary to
  - (a) Sedormid intoxication—1 case
  - (b) Gold intoxication—1 case
  - (c) Novarsenobenzol intoxication—1 case
  - (d) Monocytic leukemia—1 case
  - (e) Banti syndrome—1 case

From The John Burford Carhill Pathological Laboratories Westminster Hospital School of Medicine  
London England

## (II) Combined Form of Purpura

Aplastic anemia thrombocytopenia and hypoprothrombinemia

(a) Idiopathic form—1 case

(b) Novarsenobenzol intoxication—1 case

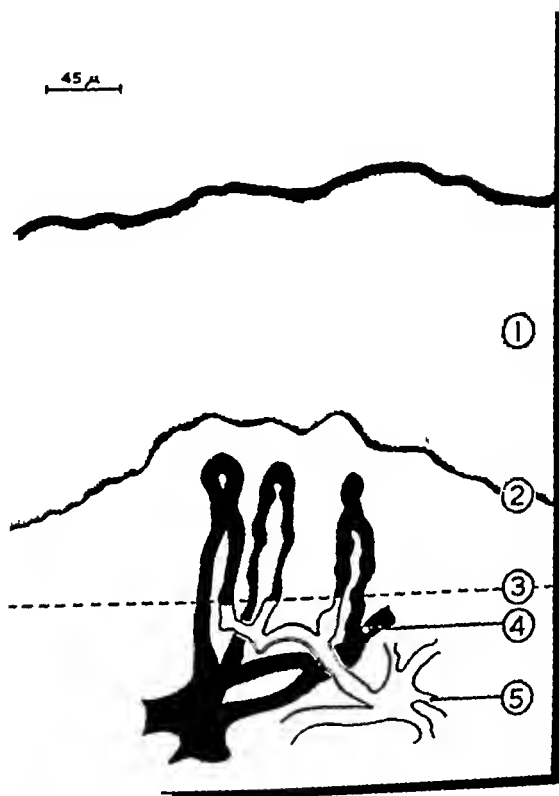


FIG. 1 Simplified section of skin to show the nature and position of the terminal vessels. The dotted line indicates the position at which the petechiae form. Only the vessels in solid black can be seen by the microscope (1) horny layer of skin, (2) malpighian layer, (3) level of petechial hemorrhage formation, (4) 1st collecting venule (5) terminal arteriole

## (III) Non Thrombocytopenic Purpura

(a) Anaphylactoid purpura—2 cases

(b) Essential hypoprothrombinemia—1 case

(c) Potassium iodide sensitivity—1 case

(d) Scurvy—1 case

(e) Malignant hypertension—1 case

The hematologic features of the cases are summarized in table 1

In essential thrombocytopenic purpura the exudation of blood occurs at first as a shower of red cells, which can be seen to be hurled from the vessels, and which travel at least three times the diameter of the vessel before they are brought to rest

There is no breach of the blood stream in the capillary, nor is it obliterated by pressure of the effused blood. The segment of vessel through which the red cells pass is very small in length, and the exuded cells form a thin disc, which later extends superficially to form a conical lesion around the arteriolar end of the capillary. The effused red cells are taken up by the skin lymphatics only very slowly (fig 2, 1-4).

*Secondary thrombocytopenic purpura* In the Sedormid and Gold and N A B intoxication cases studied, the lesions formed quickly and there was evidence that the red cells were rapidly taken up by lymphatics, as fine columns of red cells formed from the edges of the disc and rapidly extended.

*Monocytic leukemia* In this case the blood left the vessel very rapidly indeed, and length of affected vessel was greater, for the effused blood formed a much thicker disc. It was rapidly, almost immediately, taken into the lymphatics.

*Anaphylactoid purpura* In the two cases studied exudation was much more diffuse and there was considerable effusion of fluid as judged by the rapidity in which the details became obscured by edema. Lymphatic absorption is immediate. The iodide intoxication gave a similar picture.

*Essential hypoprothrombinemia* This case was characterized by the curious way the effused blood tracked superficially around the capillary loop, with very little lateral extension of the lesion. Lymphatic absorption was very slow.

*Aplastic anemia* Both cases reacted similarly. Two forms of lesion were produced, a large, rapidly forming hemorrhage which formed so rapidly that detailed observation was not possible, and a smaller lesion which resembled those seen in essential thrombocytopenia. Lymphatic absorption was very slow.

*Scurvy* The petechiae which formed were of two types, (a) large (up to 2 mm in diameter) and (b) small in size. The small lesions formed in the usual way from the arteriolar end of a single capillary loop, the large lesions formed similarly from a cluster of adjacent capillary loops, usually three in number, apparently arising from a common arteriolar twig. These lesions rapidly became confluent and the combined lesion spread rapidly. The effused blood was quickly taken up by the lymphatics.

*Malignant hypertension* The lesions here did not appear until the constricted capillary loops were fully dilated. The lesions spread rapidly and tended to be confluent (fig 3). Absorption was moderately rapid.

#### COMMENT

It will be noted that despite the different causes for the purpuric manifestations, the lesions produced all lay in the same segment of vessel, the arteriolar end of the capillary loop. In no case were lesions found elsewhere.

It would seem under the conditions of this test that this small segment of the vessel is unable to prevent the escape of red cells from the lumen. It has been shown by McFarlane<sup>2</sup> that the nailfold capillaries in various kinds of purpura display abnormal reactions to puncture with a quartz fibre, in that the capillary loop is unable to contract as do normal capillaries under similar stimuli. The part of the capillary from which the red cell exudation occurs is, moreover, that part

TABLE 1—Hematological Features of 17 Cases of Hemorrhagic Disease

No	Diagnosis	Age and sex	Duration of symptoms	Nature of lesions	Bleeding Time	Coagulation time	Platelet count per cu mm	Prothrombin index %	Treatment and sequel
1	Essential thrombocytopenia	23, F	Age 2-6 and 21-23	Epistaxis	10 min ++	3 min (venous)	25,000	—	Splenectomy age 6 Recurrence age 21 Symptomatic
2		23, F	9 mo	Menorrhagia	10 min +++	3 min 10 sec capillary	20,000	—	Splenectomy apparent cure
3		24 M	6 mo	Purpuric rash on trunk	10 min ++	2 min (capillary)	1,700-62,000	—	Blood transfusion refused Splenectomy
4		45, M	23 yr	Purpuric rash on abdomen	10 min ++	2 min 5 sec (capillary)	15,000-20,000	—	Splenectomy Died 3 days later of coronary thrombosis
5		33, M	27 yr	Purpuric rash—no inconvenience	10 min +	8 min 30 sec (venous)	2,500	100	Nil
6	Scleroid	72, F	2 da	Purpuric rash Hematuria	12 min +	2 min 30 sec (capillary)	3,300	—	Death from coronary disease
7	Gold	55, F	2 wk	Epistaxis	10 min ++	8 min 40 sec (venous)	25,000	—	Recovered after two transfusions of blood
8	N A B	47, F	5 da	Bruises on legs Melena Menorrhagia	10 min ++	8 min 10 sec (venous)	4,300	—	Many transfusions Recovery
9	Monocytic leukacemia	73 M	5 wk	Bruises on arms	10 min ++	8 min (venous)	13,600	—	Died
10	Banti syndrome	51 F	All life	Bruising especially after operations	10 min ++	7 min (venous)	55,000	85	Splenectomy Improved
11	Aplastic anemia	50 F	6 wk	Vaginal hemorrhage	10 min ++	16 min 25 sec (venous)	43,000	42	Died

12	Aplastic N A B	51, M	3 mo	Purpura legs, anemia	10 min ++	11 min (venous)	3,400	73	Many transfusions covered
13	Anaphylactoid purpura	5, M	3 wk	Massive bruises of buttock	1 min 10 sec	4 min 45 sec (venous)	25,000	100	Improved following ton sillectomy
14		45, F	9 mo	Petechial rash on arms	3 min	8 min (venous)	271,000	100	Splenectomy Somewhat improved
15	Essential hypoprothrombinemia	26, M	25 yr	Epistaxis	10 min ++	9 min lowest 12 min highest (venous*)	213,000 230,000	66 74	Occasional transfusions
16	KI sensitivity	67 M	1 wk	Purpuric rash on arms—legs	3 min	9 min 30 sec (venous)	265,000	—	Removed from KI Died 3 months later carcinoma
17	Scurvy	58, M	1 wk	Hemarthrosis of ankle Purpuric rash on leg	2 min 5 sec	9 min (venous)	293,000	100	Saturated with ascorbic acid after 1 Gm for 6 days
	Normal	—	—	—	1-3 min	2-5 (capillary) 5-12 (venous)	200,000- 350,000	100	

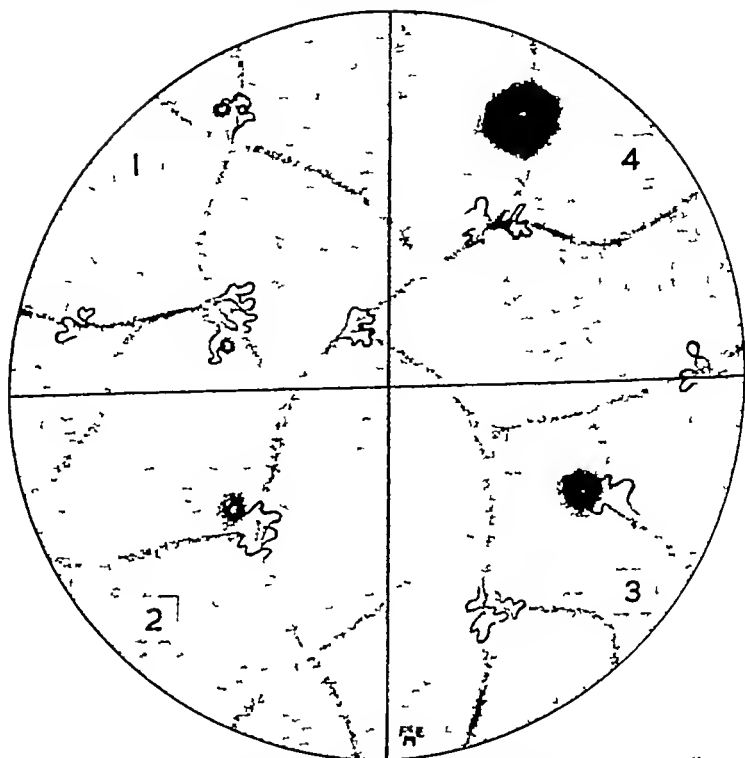


FIG 2. Simplified drawing of the terminal vessels as seen through the microscope to illustrate the formation of a petechial haemorrhage in essential thrombocytopenia purpura

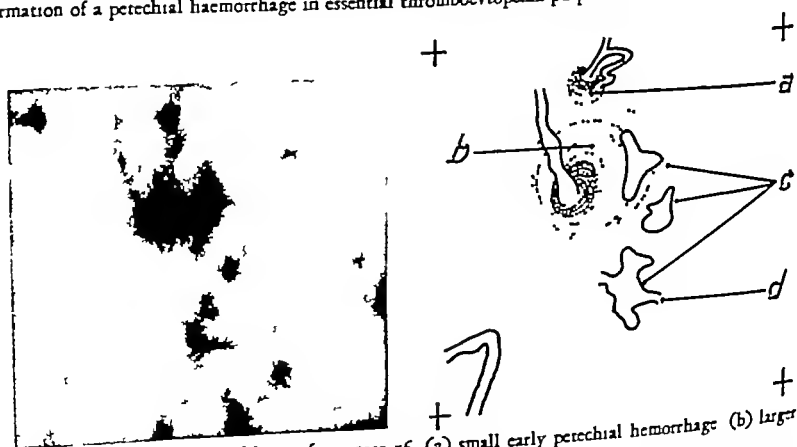


FIG 3. Photomicrograph of lesion from case 16 (a) small early petechial hemorrhage (b) larger lesion (c) dilated capillary loops (d) relatively constricted loop

from which fluid leaves the vessels for the tissues normally Landis' showed by direct measurement that the intracapillary pressure at this point is higher than

elsewhere in the loop. Furthermore, the lesions occur at a point where the tightly constricted precapillary arteriole dilates suddenly to form the capillary loop. It is evident that this arteriolar-capillary junction is of great importance in the maintenance of blood flow and the nutrition of tissues generally. It is tempting to postulate that a selective poisoning of this junction could produce hemorrhages in the mucosae and also cause thrombocytopenia by an upset of megakaryocyte nutrition. The poison must leave the circulation for the tissues at the point described, and the cells of the vessel wall must be thus exposed to a much greater selective concentration than elsewhere. This theory can be applied to essential thrombocytopenic purpura on the assumption that the spleen produces a toxic substance. It explains the prompt cessation of hemorrhage immediately following splenectomy in this disease. Similar explanations can be deduced to fit other hemorrhagic syndromes and diseases.

#### SUMMARY AND CONCLUSIONS

- 1 The capillary resistance test has been studied by a special technic of capillary microscopy
- 2 Seventeen cases of hemorrhagic diseases of differing etiology have been thus studied
- 3 The site of capillary hemorrhage has been localized to the arteriolar end of the capillary loop
- 4 Selective damage to the arteriolar-capillary junction will explain many types of hemorrhagic syndromes

#### ACKNOWLEDGMENT

I wish to thank the honorary staff of the Westminster Hospital for permission to study cases under their care. I am much indebted to Dr P. Hansell and Miss F. E. McAdoo of the Westminster Hospital Department of Medical Photography for assistance with the illustrations.

#### REFERENCES

- <sup>1</sup> LANDIS E. M. *Heart* 15: 209, 1930
- <sup>2</sup> MACFARLANE R. G. *Quart. J. Med.* 10: 1, 1941



## EDITORIAL

AND NOW B<sub>12</sub>

IN THESE crowded days when one therapeutic miracle succeeds another in rapid succession, the appearance of a new substance with almost incredible therapeutic effects inspires but little excitement. Successive triumphs by teams of chemists, often working in commercial laboratories, appear to have left us jaded. The isolation of vitamin B<sub>12</sub> in the research laboratories of Merck and Company in this country<sup>1</sup> and almost simultaneously in the Glaxo Laboratories in England<sup>2</sup> is the most recent case in point. Here is a substance that, when given to a patient suffering from pernicious anemia, results in a maximal reticulocyte response and a near maximal erythrocyte response following a single injection of 5 to 10 thousandths of a milligram (0.00005 Gm.)<sup>3</sup> Has there ever been in the history of medicine a more potent material, microgram for microgram?

Folic acid (pteroylglutamic acid) came out of the research laboratories of the Lederle Laboratories. Its history has been told in these columns.<sup>4</sup> Folic acid and the folic acid antagonists<sup>5</sup> will long stand as a monument to Dr. Yellapragada SubbaRow, who initiated work with these materials and carried it along brilliantly.

It is of interest that a bacterium was used as the assay animal in testing both these materials. With folic acid, *Lactobacillus casei* was used, with vitamin B<sub>12</sub>, it was the *L. lactis* Dorner.<sup>6</sup> Successive assays of concentrated and reconcentrated material required a readily available means for assay and this the bacterium supplied, since the necessary growth factor proved to be identical with the liver extract factor required by the human in erythropoiesis.<sup>6</sup>

Search for the factor in liver extract that is responsible for its hematopoietic and neurologic effects has proceeded almost continuously since liver was first found to be effective in the treatment of pernicious anemia. A year after the introduction of liver, Cohn<sup>7</sup> in 1927 produced a liver extract called Fraction G, this was a water soluble material obtained after protein precipitation. From this substance a solution was later prepared for parenteral use, at first in crude form containing only 1 or 2 units per cc. of extract, and later in concentrated form containing 10-15 units per cc. The concentrated extracts proved to be of greatest value since in a small amount of solution they gave maximal effects with the least local irritation. They were furthermore highly potent in combating and preventing neurologic involvement.

The place of the crude liver extracts in therapy became quite limited, particularly with the advent of folic acid. This latter material, although only partially helpful in typical Addisonian pernicious anemia was of distinct value in other (atypical) members of the pernicious anemia family, i.e., in sprue, tropical macrocytic anemia, pernicious anemia of pregnancy and megaloblastic anemia of infancy.<sup>8</sup> Here the response was often better than with liver extract and neurologic involvement did not occur.

The mysterious relationships between folic acid and liver extract, which are as yet by no means solved, will probably become better understood now that chemi-

cally pure vitamin B<sub>12</sub> is at hand. At this writing, B<sub>12</sub> appears to be the long-awaited liver extract factor. In minute amounts it appears to possess all the effects of liver extract, both hematologically and neurologically. That it acts on the neurologic disturbance would tend to discredit the assumption that the hematologic and the neurologic lesions of pernicious anemia are due to separate deficiencies.

Some of the macrocytic deficiency states may conceivably not be benefited by B<sub>12</sub> administration. This may indicate that the pernicious anemia family of diseases is composed of a group of different types of deficiency states but characterized by the common denominator of a megaloblastic bone marrow and macrocytic anemia. In one group are those cases primarily benefited by liver extract, the other is composed of cases in which the best effects appear to be obtained with folic acid. It is reasonable to assume that in the latter group the primary deficiency is in folic acid. A working concept for the present (subject to change at a moment's notice) is as follows:

<i>Pernicious Anemia Family (Megaloblastic Bone Marrow with Macrocytic Anemia)</i>	
<i>Deficiency in Vitamin B<sub>12</sub></i>	<i>Deficiency in Folic Acid</i>
Addisonian Pernicious Anemia	Sprue (certain cases)
	Tropical Macrocytic Anemia
	Refractory Megaloblastic Anemia
	Pernicious Anemia of Pregnancy
	Megaloblastic Anemia of Infancy
Sprue (certain cases)	

It should be noted that the syndromes in which folic acid is most effective include largely those conditions in which free hydrochloric acid is present in the gastric juice. In the presence of complete achlorhydria, as Spies<sup>8</sup> has already postulated, folic acid does not protect against neurologic involvement. Already, there is indication that B<sub>12</sub>, like liver extract, may be ineffective in the pernicious anemia of pregnancy, whereas folic acid is highly effective.<sup>9</sup> Further investigations will undoubtedly bring a more complete elucidation of the different types of deficiency states with macrocytic anemia.

Although the chemical formula of vitamin B<sub>12</sub> has not as yet been announced, it may be presumed that work on this problem as well as on methods for synthesis is going on. In solution, B<sub>12</sub> has a purplish hue and the startling discovery has been made by both the Glaxo and Merck Laboratories that this is due to the presence of cobalt.<sup>10</sup> For years, cobalt has been used in the experimental production of polycythemia.<sup>11</sup> The epizootic occurrence of cobalt deficiency in sheep and cattle in Australia, New Zealand, Canada, and even in this country has been reported.<sup>11</sup> Animals so affected have developed anemia, changes in coat, weakness, emaciation, and finally death as the result of the cobalt deficiency. The whole subject of cobalt metabolism and of the activities of this trace element in the human economy is thus thrown wide-open for new vistas of research.

Vitamin B<sub>12</sub> may prove to play a prominent role not only in therapeutics but also in the field of animal nutrition. It has been recognized for a considerable period that an unknown substance or substances present in crude materials such as fish meal, cow manure and liver is required for optimum growth of chicks and for

adequate hatchability of hen's eggs.<sup>10</sup> Recently, the administration of a concentrate of this animal protein factor prepared by Stokstad et al.<sup>11</sup> in two cases of pernicious anemia produced a well defined hematopoietic response. More recently, Ott and collaborators<sup>12</sup> demonstrated that crystalline vitamin B<sub>12</sub> can replace these crude sources of the animal protein factor in promoting chick growth. Thus, B<sub>12</sub> may be responsible either wholly or in part for the growth promoting activity of such feed supplements.

The finding of a potent growth factor in cow manure and doubtless in the excreta of other animals harks back to the days of the witches' brew, and to the naive medicine of certain country districts. Perhaps there was something in these old concepts of medical therapy after all!

In this complex modern world where chemists and physicists and mathematicians are constantly at work, one never knows what new complexities lie ahead of us, what new worlds await us in tomorrow's news!

WILLIAM DAMESHEK, M.D.

#### REFERENCES

- <sup>1</sup> RICKES, E. L., BRINK, N. G., KONIUEZY, F. R., WOOD, T. R., AND FOLKERS, KARL. Crystalline vitamin B<sub>12</sub>. *Science* 107: 96, 1948.
- <sup>2</sup> SMITH, E. L. *Nature* 161: 638, 1948.
- <sup>3</sup> Editorial. Folic acid. *Blood* 1: 357, 1946.
- <sup>4</sup> Editorial. Folic acid, pernicious anemia and pendulums. *Blood* 3: 699, 1948.
- <sup>5</sup> Editorial. The use of folic acid antagonists in acute leukemia. *Blood* 3: 1057, 1948.
- <sup>6</sup> SHORR, MARY S. Activity of vitamin B<sub>12</sub> for the growth of *Lactobacillus lactis*. *Science* 107: 397, 1948.
- <sup>7</sup> WEST, RANDOLPH. Activity of vitamin B<sub>12</sub> in Addisonian pernicious anemia. *Science* 107: 398, 1948.
- <sup>8</sup> COHN, E. J. et al. The nature of the material in liver effective in pernicious anemia. *J. Biol. Chem.* 74: 49, 1927.
- <sup>9</sup> Editorial. J. A. M. A. 138: 595, 1948. Anti-anemic properties of vitamin B<sub>12</sub>.
- <sup>10</sup> BETHELL, FRANK. Personal communication.
- <sup>11</sup> RICKES, E. L., BRINK, N. G., KONIUEZY, F. R., WOOD, T. R., AND FOLKERS, KARL. Vitamin B<sub>12</sub> and cobalt complex. *Science* 108: 134, 1948.
- <sup>12</sup> Quoted by CARTWRIGHT, G. E. Dietary factors concerned in erythropoiesis. *Blood* 2: 256, 1948.
- <sup>13</sup> HAMMOND, J. C. Dried cow manure and dried rumen contents as a partial substitute for alfalfa leaf meal. *Poultry Science* 23: 471, 1941.
- <sup>14</sup> RUBIN, M. AND BIRD, H. R. A chick growth factor in cow manure. *J. Biol. Chem.* 163: 387, 1946.
- <sup>15</sup> NESTLER, R. B. et al. *Poultry Science* 15: 67, 1936.
- <sup>16</sup> STOKSTAD, E. L. R., et al. Activity of microbial animal factor concentrates in pernicious anemia. *J. Lab. & Clin. Med.* 33: 860, 1948.
- <sup>17</sup> OTT, W. H., RICKES, E. L., AND WOOD, J. R. Activity of crystalline vitamin B<sub>12</sub> for chick growth. *J. Biol. Chem.* 174: 1047, 1948.

# ABSTRACTS

JOSEPH F. ROSE, M.D., *Editor*

ABSTRACTERS

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JAN WALDENSTRÖM, M.D., Uppsala, Sweden

## CYTOLOGY

STUDIES ON THE MEGAKARYOCYTE I THE NORMAL GRANULOPOIESIS OF THE MEGAKARYOCYTE II DEFICIENT GRANULOPOIESIS IN THE MEGAKARYOCYTE IN ESSENTIAL THROMBOCYTOPENIC PURPURA *E. Schwarz* From Department of Hematologic Research, Michael Reese Hospital, Chicago, Ill. Arch. Path. 45: 333-353, 1948

This series has as background Schwarz's experience since 1888, when he published his first paper and his chief preoccupation was with morphology and biology, to his recent freedom from clinical and teaching duties and a return to studies in cytology. His contribution to the megakaryocyte problem is chiefly a focusing of attention to the previously neglected developmental history of the granulation and the light areas in the cytoplasm. After becoming thoroughly acquainted with the appearance of early developmental forms of normal megakaryocytes, his studies were carried over to cases of Werlhof's essential thrombocytopenic purpura. In megakaryoblasts and early promegakaryocytes the first evidence of granulopoiesis appears in a light staining area located close to the nucleus. Because this area increases and becomes pinkish with the growth and development of the cell, Schwarz has designated it the "functional area." In the light of some recent studies on the cytoplasm of immature blood cells, the proposed term is particularly satisfactory because it probably represents the negative images of underlying cytoplasmic organoids and as such justifies the importance which Schwarz has assigned to it. Under normal conditions, the functional area is related to granulopoiesis. But under pathologic conditions, as in some cases of essential thrombocytopenic purpura, the functional area does not produce azurophilic granules and it may even become hyalinized. According to Schwarz's analysis of megakaryocytes in essential thrombocytopenic purpura, these cases fall into three groups: those with intact granulopoiesis, which are the common type; those with functional disturbance of granulopoiesis, and those with degeneration and destruction of megakaryocytes. The type with hyalinization of the functional area may be due to deficiency of some substance necessary for megakaryocytic granulopoiesis. It is to be regretted that this important article was not illustrated with colored plates.

O P J

CONTRIBUTION TO THE PATHOLOGY OF THROMBOCYTOGENESIS *F. Hefmanský* From the Department of Medicine, Hospital of Charitable Sisters, Prague. Čas. lek. čes. 86: 232, 1947

In accordance with Jasínský (Schw. med. Wschr. 12:18, 1944) the megakaryocytes were classified into six groups, namely megakaryoblasts, promegakaryocytes, basophilic megakaryocytes, transitional forms, granular megakaryocytes and nude nuclei.

In five cases of thrombocytopenia the megakaryocytic formula was the following:

CASE 1. Myelophthisic anemia due to radium with leukopenia and thrombocytopenia. FORMULA

0-1-3-15-77-4

CASE 2. Acute thrombocytopenic purpura. FORMULA 2-12-13-24-48-0

CASE 3. Recurrent essential thrombocytopenia. FORMULA 1-3-7-16-72-1

CASE 4. Essential thrombocytopenia. FORMULA 0-2-5-8-79-6

CASE 5. Hypersplenic thrombocytopenia in splenomegalic cirrhosis. FORMULA 1-4-5-6-81-3

No relation could be found between the intensity of the morbid state, the appearance and num

platelets and the distribution formula of megakaryocytes in the bone marrow, nor was hypersegmentation or vacuolization of megakaryocytes in any way connected with the specific thrombocytopenic syndrome. In many cases of disturbed thrombocytopoiesis no changes at all could be observed in the megakaryocytes in the bone-marrow.

M.N.

**INDIVIDUAL CELLS UNDER PHASE MICROSCOPY BEFORE AND AFTER FIXATION** R. Buchsbaum. From Institute of Radiobiology and Biophysics, The University of Chicago. Chicago, Illinois. *Anat. Rec.* 122: 19-36, 1948.

The use of phase microscopy makes possible a method by which the cytology of living and dying cells can be studied without the interference of fixatives. Zollinger has recently reported changes observed of tumor cells *in vivo* and *in vitro* with the phase microscope (*Am. J. Path.*). Buchsbaum has utilized a similar approach to determine which fixatives yield preparations most representative of the living cell. His studies were limited to salamander macrophages grown in tissue cultures. Certain fixatives like absolute alcohol, Carnoy's and Bouin's solutions distort the cytoplasm more than the nucleus. Formol alone in alkaline solution was a better fixative than either of these and better than formol in acid solution. The best general fixatives were Zenker formol and Zenker formol-osmic, the latter being the better of the two. Although phase microscopy alone has not revealed any new structure which had not been preserved by the better fixatives, it does offer a means of checking the rationale for using them.

O.P.J.

**LOCALIZATION OF LIPIDS AND OTHER CHEMICAL SUBSTANCES IN THE MAST CELLS OF MAN AND LABORATORY MAMMALS** W. Montagna and C. R. Noback. From Department of Anatomy, Long Island College of Medicine, N. Y. *Anat. Rec.* 100: 535-546, 1948.

Because there is convincing evidence that tissue mast cell granules contain heparin, quite a few articles have been published recently dealing with observations on the chemical cytology of these cells. The present article extends this knowledge by demonstrating that mast cell granules contain phospholipin, peroxidase and lipase.

O.P.J.

**ASPIRATION OF BONE MARROW FROM THE ILIAC CREST: COMPARISON OF ILIAC CREST AND STERNAL BONE MARROW STUDIES** M. A. Rubinstein. From the Montefiore Hospital, New York. N. Y. *J. A. M. A.* 137: 1281-1285, 1948.

The author of this article has performed over 1000 aspirations of the iliac crest bone marrow, and presents his findings and technique with the thesis that marrow puncture, when indicated, can be performed easily, safely, and advantageously at the iliac rather than the sternal region. In 216 of the 1000 patients comparative studies were done on samples of marrow obtained simultaneously from the two sites; the normal marrow picture was identical at both locations and when the marrow was abnormal the pathologic alterations usually occurred in parallel fashion in both areas.

The advantages of the iliac crest over the sternum, according to the author, include (1) safety, since no vital organs underlie the ilium; (2) ease of performance, by virtue of less pain and less apprehension than at the sternum; and (3) ease of repetition.

Of especial interest were those diseases of the marrow in which there was patchy involvement of the bone: certain leukemias, osteosclerosis, myeloma, neoplastic infiltration. In several such cases diagnosis was made by iliac aspiration after a sternal puncture was fruitless. (In others the reverse was true.) The iliac bone bears no special virtue in such cases, presumably, in such diseases marrow aspiration at various portions of the sternum and at the spinous processes—as well as at the iliac crests—may be required to obtain diagnostic information. The statement, therefore, that in a number of cases of malignant infiltration of the bone marrow neoplastic cells were seen more often in the iliac than in the sternal aspiration is somewhat startling and will bear substantiation.

The ilium, spinous processes, and sternum are now commonly used sites for puncture aspiration of the bone marrow. In selected cases multiple punctures in these sites may prove of value over single punctures at any one site. (See Loge, *Blood* 3: 198, 1948, and Dameshek, *Blood* 3: 209, 1948).

S. E.

## ERYTHROCYTES AND ERYTHROCYTIC DISEASE

CHRONIC HAEMOLYTIC ANEMIA WITH HAEMOGLOBINURIA THE MARCHIAFAVA MICHELI & SYNDROME M D Hickey and L. K. Malley From the Mater Misericordiae Hospital, Dublin Quart J Med 17 1 1948

The 49 year old man whose case history is reported in this paper showed nocturnal hemoglobinuria for a period of ten days beginning twenty four days after multiple transfusions. He exhibited persistent hemoglobinuria without diurnal variation during the oral administration of iron but at other times he was free of hemoglobinuria.

Serum heated to 56 C. was shown to have an inhibitory effect on hemolysis of the patient's cells in vitro, and the intravenous administration of 400 cc. of heated serum was followed by a cessation of hemoglobinuria for thirty-six hours.

The number of cells susceptible to acid hemolysis was computed and correlated with the effect of transfusion and the reappearance and cessation of hemoglobinuria. However, judging from the data presented it is evident that the occurrence of hemoglobinuria is influenced by some factor in addition to the number of susceptible cells present.

R.S.E.

TRUE PERNICIOUS ANEMIA WITHOUT ACHLORHYDRIA Alex Murphy M J Australia 1 521, 1948

The finding of free hydrochloric acid in the gastric secretion of a 23 year old girl who appeared to have typical Addisonian pernicious anemia led the author to attempt to satisfy all criteria as to diagnosis and to exclude other types of macrocytic anemia. The subject appeared to have pernicious anemia because of a typical reticulocyte response to refined liver extract followed by a rise in the erythrocyte count and hemoglobin. The only atypical finding was an MCHC of 27.6 per cent which is not explained. Later, a relapse was induced by withdrawing liver which resulted in reappearance of anemia and macrocytes. Biologic assay demonstrated a lack of extrinsic factor in the gastric contents. The failure to find megaloblastic change in the marrow makes the case not quite complete but is readily explained since marrow examination was not made until seventeen days after treatment. Megaloblastic changes did not occur during the partial relapse. The author believes that it is possible to conclude that true pernicious anemia can and does occur in persons with free hydrochloric acid in their gastric juice and therefore that achlorhydria is not essential to the development of true pernicious anemia.

R.S.E.

ANOMALIES OF THE INTESTINAL ABSORPTION OF FAT II THE HAEMATOLOGY OF IDIOPATHIC STEATORRHEA W T Cooke, A C Frazer, A L P Peeney H G Sammons and M D Thompson From the Queen Elizabeth Hospital and the Department of Medicine and Pharmacology Birmingham University Quart J Med 18 9-23 1948

Studies of the peripheral blood particularly the red cell morphology of idiopathic steatorrhea are reported. The most consistent abnormalities were the increase in mean cell diameter, an increase in the diameter thickness ratio and an increase in resistance to hemolysis in hypotonic saline. The mean corpuscular hemoglobin concentration was below normal in most instances. In 4 of 17 cases studied the sternal marrow was indistinguishable from that of pernicious anemia. In the remaining 13 the sternal marrow showed a mixture of iron deficiency normoblasts and large atypical normoblasts. Fecal urobilinogen was increased in 5 of 11 patients studied. There was no consistent response to therapy with refined and crude liver B complex iron and a variety of other agents. The authors discount the similarity of the anemia of idiopathic steatorrhea to pernicious anemia and the unitarian theory of the etiology of macrocytic anemias in general, a concept which in its strict interpretation has already been challenged by the discovery of the Wills factor.

The authors conclude that the similarity of the anemia of idiopathic steatorrhea to pernicious anemia is largely superficial.

R.S.E.

THE NORMAL RED CELL IN INFANCY AND CHILDHOOD SOME RECENT ADVANCES B Dittus and I J Wolman From The Children's Hospital of Philadelphia (Department of Pediatrics School of Medicine University of Pennsylvania) Am J M Sc. 215 694-709 1945

This article discusses in a general way the structure of the red cell and hemolytic mechanisms with special reference to osmotic resistance. Fetal erythropoiesis and normal red cell values in infancy and childhood are also reviewed. References are well chosen and bring the subjects dealt with up to date relating to pediatric hematology.

C.A.F.

OBSERVATIONS ON ANEMIAS IN STARVATION O. Seyka From the District Hospital L 124 in Terežín ghetto Čas lékař čes 86 583, 1947

These observations were made on prisoners in concentration camps in Terežín Bohemia. Under extremely difficult conditions, the author succeeded in performing blood examinations in 50 healthy male prisoners, the red blood cells were almost normal in number but they were distinctly macrocytic with color index of 1.2 to 1.3. These blood examinations were supervised by Professor Hirschfeld who himself, was one of the prisoners. This macrocytosis seems to have been conditioned by a deficiency in amino acids and vitamins, but no definite conclusions could be reached in view of the impossibility of exact scientific investigation.

M.N.

RETICULOCYTES EXAMINED BY THE DARKFIELD METHOD F. Lebert From the 3rd Medical Clinic, Charles University Prague Čas lékař čes 86 11, 1947

Reticulocyte studies were made by the darkfield method described by A. Nixet (Acta med Scand 1944). The identification of reticulocytes by the darkfield method of microscopy was very easy, the granulofilamentary substance appearing in the form of spots and threads of varying size and of yellow greenish hue.

Fifty healthy young men and women between 18 and 38 years were examined by this method, the percentage of reticulocytes was higher than with the usual method using 1 per cent solution of brilliant cresyl blue (darkfield 5 to 36 per mille, brilliant cresyl blue 0.5 to 14 per mille).

M.N.

DEVELOPMENT OF HEINZ BODIES M. Rejsek From the Clinic of Occupational Diseases, Charles University Prague Čas lékař čes 86 1183, 1947

The development of Heinz bodies could easily be followed in rabbits poisoned by dinitrobenzene. This toxic agent administered to the animals in the daily dose of 20 mg/Kg, provoked a hemolytic anemia with a steady decrease of hemoglobin and the red blood cells so that by the seventh day the number of red blood cells fell to one fifth of the original count. Heinz bodies appeared in the red cells as soon as the second day, they were attached to the surface of the cell by a kind of pseudopod which finally disappeared and the Heinz body was set free to circulate in the blood stream. Nile blue sulfate was the best dye for the supravital staining of the Heinz bodies.

M.N.

PLASMA IRON IN BLOOD DISEASES L. Donner From the 2nd Medical Clinic, Charles University, Prague Čas lékař čes 86 111, 1947

Plasma iron determinations were made in patients suffering from various blood dyscrasias. Decrease of plasma iron was found in acute or chronic blood loss (37 cases), in chlorosis (1 case), in hypochromic anemia (5 cases), in polycythemia (5 cases) and in chronic leukemia (8 cases), increase of plasma iron was found in aplastic anemia (6 cases) and in pernicious anemia (26 cases).

Influence of treatment on plasma iron was very marked in some cases, increase could be observed in chronic leukemia and in polycythemia following x-ray therapy, decrease to subnormal values occurred in pernicious anemia following liver therapy.

M.N.

FOLIC ACID THERAPY, ITS EFFECT AS OBSERVED IN TWO PATIENTS WITH PERNICIOUS ANEMIA AND NEUROLOGIC SYMPTOMS. By S. D. Jacobsen, L. Berman, A. R. Axilrod and E. C. Vander Hide From Wayne University College of Medicine and City of Detroit Receiving Hospital, and the Anemia Laboratory Harper Hospital Detroit Michigan J. A. M. A. 137 825-827, 1948

This is an additional report emphasizing the occurrence of neurologic relapse in patients with pernicious anemia under treatment with folic (pteroylglutamic) acid. Two patients aged 78 and 62 respectively, showed good hematologic, clinical, and, initially, neurologic remissions during treatment with folic acid. In both instances, numbness and tingling of the extremities improved, and in the second case where drowsiness, confusion, and irrationality were present, these symptoms also disappeared. On maintenance doses of folic acid, however (10 mg daily by mouth) paresthesiae recurred, and these and other neurologic symptoms and signs progressed despite increase in the dosage of the drug. Liver extract was ultimately given instead to each patient with apparently satisfactory response.

It is of interest in these, as in other similar cases, that the paresthesiae initially present disappear on treatment with a drug which subsequently allows their redevelopment. One wonders whether originally the paresthesiae were not, perhaps, on a noncentral basis, and that at the time of their recurrence—together with vibratory sensation changes, position sensation changes etc—they are due to a lesion different from that which caused them initially. At any rate liver extract and not folic acid alone, seems necessary for the satisfactory clinical treatment of such cases.

S E

## HEMOGLOBIN AND HEMOGLOBIN METABOLISM

THE EFFECT OF STROMA FREE HAEMOGLOBIN ON THE ISCHAEMIC KIDNEY OF THE RABBIT. *A W Badenoch and E M Darmady*. From the R. A. F. Hospital, Wroughton, England. Brit J Exper Path 29 215-223 1948

The authors conducted a series of experiments in rabbits combining right nephrectomy and left renal artery occlusion with or without the subsequent injection of stroma free hemoglobin. The results show that hemoglobin per se is not toxic and that renal damage must precede a detrimental effect from either hemoglobin or its derivatives.

O P J

CYANOSIS IN TREATMENT WITH SULFONAMIDES. *W Henbier and M Kesse*. From the University Institute of Pharmacology, Berlin (Germany). Schweiz med Wchnschr 77 1337-1339 1947

The authors critically discuss recent publications and maintain their formerly given opinion that methemoglobin and sulfhemoglobin have to be considered as the primary reason for cyanosis in sulfonamide treatment. They agree, though, that under special conditions further causes may intervene.

C.M

EVALUATION OF SOME METHODS OF HEMOGLOBIN DETERMINATION. *M. Rejškova and K. Rejšek*. From the Clinic of Occupational Diseases, Charles University, Prague. Čas lék čes 86 237, 1947

The results of hemoglobinometry, obtained with Sica hemometer were compared with those of Sahli's acid hematin method and of the photometric procedure of Heilmeyer Mutius, with the Sica hemometer and the photometric procedure. oxyhemoglobin is reduced to hemoglobin by sodium hydro-sulfite.

Sica hemometer proved to be the most reliable apparatus of hemoglobinometry. It was more exact than the procedure of Heilmeyer and Mutius. Sahli's method using acid hematin is very unreliable and should be discarded from scientific laboratory work.

The results obtained with any one of the procedures were compared according to statistical methods.

M.N

SOME PHYSICO-CHEMICAL PROPERTIES OF THE BLOOD BILIRUBIN. *M Netoušek*. From the Medical Department State Hospital Motol, Prague. Čas lék čes 86 799 1947

The solubility of the so-called indirect bilirubin in chloroform was discovered by Drer and the author in 1927. Independently of Yllpö (1913) and Grunenberg (1923) the nature of this phenomenon has not yet been elucidated.

In cancerous sera bilirubin may be soluble in ether. This unusual property of the blood bilirubin observed first by Ascoli (1935) and Albers and Merten (1940) has been found to be fairly constant and may be of some use in discriminating calculous and cancerous obstruction of the common bile duct. Further studies in this direction are desirable.

M.N



## BLOOD TRANSFUSION

**EXSANGUINATION TRANSFUSION IN THE TREATMENT OF ERYTHROBLASTOSIS FETALIS** *A. Raška and A. Bernard*  
From the State Health Institute and the Maternity Department of the City Hospital Prague Čas  
lék čes 86 1517 1947

A description of three successfully performed exsanguination transfusions is given. All were Rh positive infants born of Rh negative mothers and free Rh antibodies could be demonstrated in their sera in a high titer, the sera of their mothers contained Rh antibodies in a high titer as well.

A simple syringe technic was used in these transfusions. Native blood of the donor was given into the left saphenous vein (in the third case also into the cubital vein) and the blood was let out from the opened right radial artery or its branch. In the first case 430 cc. of blood were transfused and 300 cc. of blood were withdrawn; as this proved not to be sufficient the transfusion was repeated so that a total of 880 cc. of blood were transfused and 470 cc. withdrawn. In the second case 450 cc. of blood were transfused in one session and 380 cc. of blood withdrawn. In the third case, 590 cc. of blood were transfused and 430 cc. withdrawn. The transfusion lasted from 50 to 90 minutes. The results were satisfactory; all three patients did well. No heparin was used.

M N

**DENATURED VEAL PLASMA TO SUBSTITUTE HUMAN BLOOD AND PLASMA FOR TRANSFUSION PURPOSES** *J. Milka, Vlad. Rapant and B. Zapletal*  
From the Institute of Physiology and the Department of Surgery Palacky University Olomouc Czechoslovakia Čas lék čes 86 33 1947

Denatured veal plasma was prepared according to the method indicated by Massons (Lancet 2 341 1946); the denaturation of plasma proteins was effected by formalin and heat. This liquid was completely devoid of any antigenic or toxic properties; it had the same usual colloid osmotic pressure as before and did not provoke any sensitization in the recipient's body. The denatured veal plasma can therefore be considered a most perfect substitute of human blood or plasma for transfusion purposes; it is claimed.

M N

**THE BLOOD BANK IN THE STATE HEALTH INSTITUTE OF PRAGUE** *Stolzer, Štěrbová*  
State Health Institute Prague Čas lék čes 86 26, 1947

The distribution of blood groups has been determined in 6478 inhabitants of Bohemia. The results were: Group O 37.8 per cent, group A, 41.5 per cent, group B 14.1 per cent, group AB 6.6 per cent, subgroup A<sub>1</sub> 89.3 per cent, subgroup A<sub>2</sub> 10.7 per cent, subgroup A<sub>1</sub>B 70 per cent, subgroup A B 30 per cent, group M 33 per cent, group N 15 per cent, group MN 52 per cent.

Among the universal donors, only 20 per cent had a low agglutination titer.

M N

**IS PLACENTAL BLOOD SUITABLE FOR TRANSFUSION?** *V. Rešek*  
From the Maternity Hospital in Prague Čas lék čes 86 1246 1947

The author preserved and stored placental blood taken from 1000 parturient women and found the procedure to be harmless to the mother as well as to the baby. This blood was safely used in 57 transfusions performed in the hospital or elsewhere and its biologic value was found to be perfect. The same resulted from physicochemical and biologic investigations of the placental blood performed by the author. Its high content in hemoglobin, calcium and hormones makes it very suitable for transfusion purposes; the absence of isoagglutinins or their low titer makes the transfusion of placental blood a safe procedure. There is no danger of sensitization if repeated transfusions are administered. The cost of the placental blood is insignificant and the technic is simpler than that of taking venous blood.

By storage of placental blood the task of a blood donor service is made easier and the realization of a satisfactory transfusion service even in small county hospitals is facilitated. All maternity hospitals and all departments of obstetrics should store placental blood systematically and ought thus be included into the network of the transfusion service. With two thousand deliveries yearly it could be possible to store at least one hundred liters of blood and in this way the maternity hospital should be able to supply the whole district. The technic of preservation and storage of the placental blood is simple and can easily be performed anywhere.

M N

THE SURVIVAL OF THE VIRUS OF FOOT AND-MOUTH DISEASE IN BLOOD AT 37°C J B Brooksby From the Research Institute Pirbright, Surrey, England Brit J Exper Path 29 10-19 1948

During certain preliminary experiments it was noticed that the virus of foot and mouth disease survived longer in citrated blood than in defibrinated blood Brooksby has shown that the presence of calcium ions in guinea pig blood or serum hastens the inactivation of the virus at 37°C Other detalcifying anticoagulants such as sodium fluoride and potassium oxalate had the same effect as citrate in that the inactivation was prolonged The virus in heparinized and defibrinated blood behaved similarly Further studies may reveal that the effect of the Ca ion may be direct on the virus or it may be on some enzyme system

O P J

## LEUKOCYTES AND LEUKOCYTIC DISEASE

NEUROLOGICAL MANIFESTATIONS IN LEUKEMIA J Libánský and E Ponča From the 1st Medical Clinic and the Clinic of Nervous Diseases Charles University Prague Čas lěk čes 86 1244 1947

Three cases of leukemia with involvement of the C N S are reported in this paper

Case 1 A male of 36 years Severe headache for three weeks followed by impaired vision of the right eye, nausea and signs of meningeal irritation Acute myelogenous leukemia with 73.6 per cent myeloblasts was found at blood examination Autopsy revealed a tumor like infiltration of the C N S especially of the dura mater on both the convexity and the base

Case 2 A male of 21 years Intercostal neuralgia followed fourteen days later by signs of spinal compression, blood examination revealed the presence of acute myelogenous leukemia with 44 per cent myeloblasts Autopsy showed an epidural infiltration of Th<sub>II</sub> to Th<sub>VIII</sub> On the inner side of the sternum a flat tumor 7 x 4 cm was found, consisting of oxydase positive myeloid elements, this substernal tumor, as well as the epidural infiltration and the bone marrow had a definite greenish color so that the leukemia proved to be of a chloromatous character

Case 3 A man of 53 years suffering from chronic lymphatic leukemia, developed herpes zoster, the histologic examination, performed at autopsy showed a massive leukemic infiltration of the intervertebral ganglion and of the nerves supplying the area affected by the herpes zoster

The authors reviewed the literature and collected about 300 cases of leukemia showing neurologic symptomatology The neurologic lesion most frequently observed in leukemia is a spinal cord lesion resulting in paraplegia, next in frequency follows hemiplegia caused by cerebral hemorrhage and leukemia with signs and symptoms of a cerebral tumor with or without papilledema Lesions of the cranial nerves, especially the 7th bulbar paralysis damage of the Gasserian ganglion lesions of other cranial nerves peripheral nerve lesions symptomatic herpes zoster and meningeal lesions are less frequently encountered

The leukemic lesions of the C N S are due to leukemic infiltration or to hemorrhage primary degenerative changes analogous to funicular myelosis of pernicious anemia are quite exceptional and their existence would be acceptable in only some cases associated with severe anemia of long duration

M N

CHEMOTHERAPY OF NEOPLASTIC DISEASE D A Karnofsky From the Sloan Kettering Institute and Memorial Hospital N Y C New England J Med 339 1948 1 Methods of approach 226-230 2. Trends in experimental cancer 260-270. 3 Agents of clinical value 299-305

This authoritative article deals in section 1 with the various investigative approaches to treatment of neoplastic disease and with methods of evaluating the effect of test substances on tumor tissue Section 2 summarizes the evidence for the experimental use of biologic products (bacteria molds and protozoa on urine and tissue preparations vitamins hormones) cell poisons (nitrogen mustards urethane colchicine podophyllin) carcinogenic agents radioactive substances and miscellaneous compounds (dyes b-hydroxyde stilbamidine enzyme poisons)

Of practical interest is the discussion of clinical use of these agents particularly the detailed discussion of nitrogen mustard therapy of blood dyscrasias and lymphoma The author also comments on effectiveness of radioactive phosphorus urethane and Fowler's solution in this regard

C A F

TEMPORARY REMISSIONS IN ACUTE LEUKEMIA IN CHILDREN PRODUCED BY FOLIC ACID ANTAGONIST 4 AMINOPTERYL-GLUTAMIC ACID (AMINOPTERIN) *S. Farber, L. K. Diamond, R. D. Mercer, R. F. Sylvestre Jr. and J. A. Wolff* *New England J. Med.* 238: 787-793, 1948

The authors report on 16 infants and children with acute leukemia treated with aminopterin. In this group, it showed clinical, hematologic, and pathologic evidence of improvement. Detailed accounts of the 5 most favorable cases are given. The observations extend for no longer than three months after the beginning of therapy. Stomatitis with ulceration was mentioned as a toxic manifestation of the drug.

While the immediate effect of aminopterin on the course of leukemia is dramatic in some instances, the preliminary nature of this report and the severity of the toxic manifestations of this drug should be emphasized.

C.A.F.

DIFFUSE PLASMA CELL MYELOSIS. REPORT OF A CASE IN WHICH IT SIMULATED APLASTIC ANEMIA ON POST MORTEM EXAMINATION *S. E. Schwartz, B. E. Armstrong, E. Loeffler and W. Maurer*. From the Department of Pathology and the Hektoen Institute for Medical Research, Cook County Hospital, Chicago. *Ill. Arch. Path.* 45: 380-384, 1948.

If a case of multiple myeloma has a diffuse involvement of the marrow without an infiltration of other organs, the hematologic pathologist might be misled by the gross appearance at necropsy. The authors report such a case which had a diffuse myelomatosis of the marrow without any localizing lesions, tumor formation or peripheral plasmacytosis.

O.P.J.

PLASMOCYTIC LEUKEMIA *F. Leberer*. From the 3rd Medical Clinic, Charles University, Prague. *Čas. lékař.* 86: 1366, 1947.

A rare observation of plasmocytic leukemia in a man of 42 years is reported. Duration of the disease from the onset, was fourteen months. The blood picture showed a slightly macrocytic anemia, moderate leukocytosis of 38,000 and 62 per cent myeloma cells.

M.N.

TREATMENT OF MULTIPLE MYELOMA WITH STILBAMIDINE. CLINICAL RESULTS AND MORPHOLOGIC CHANGES *By I. Snapper*. From the Mount Sinai Hospital, New York, N.Y. *J. A. M. A.* 137: 513-516, 1948.

At the original time of this report (June 1947) some 35 patients with multiple myeloma had been treated with stilbamidine, a compound found to be effective in kala-azar, and originally tried in myeloma because of the common factor of hyperglobulinemia in the two otherwise unrelated conditions. Dramatic relief of pain is recorded by the author in some cases, and at least partial relief was noted in 80 per cent of his cases. There was no effect, however, on the underlying disease itself, or on its biochemical alterations (Bence-Jones proteinuria, hyperglobulinemia). As previously noted, the striking finding was the development in the cytoplasm of myeloma cells—and in these cells only—of granules which consisted of a conjugate of stilbamidine with the ribonucleic acid of these cytoplasmic granules. The specificity of the stilbamidine for such cytoplasm suggested a fundamental characteristic of the plasma cell which distinguished it from all other blood cells. The relationship in either the cause or the treatment of the disease, however, was still to be determined.

S.E.

OBSERVATIONS IN GUINEA PIGS FOLLOWING INJECTION OF SPECIFIC HEMATOPOIETIC SUBSTANCES DERIVED FROM URINE OF HUMAN LEUKEMIC SUBJECTS. *A. Sawitsky and L. M. Meyer*. From Department of Therapeutics, N.Y.U. College of Medicine, New York City. *Am. J. Path.* 44: 1117-1135, 1948.

Extracts of urines from patients with myeloid or lymphoid leukemia were prepared by chloroform extraction. These were separated into carbinols and noncarbinols by succination. Ether was used in a second type of extraction. Guinea pigs were injected either subcutaneously or intramuscularly. Examination of lymph nodes, spleen, liver, adrenals, kidneys, lung and bone marrow showed varying degrees of hyperplasia and infiltration depending upon the extract. Carbinol (lymphoid) extracts produced a specific

lymphoid reaction and noncarbinol (myeloid) extracts produced a specific myeloid reaction. The results of these experiments justify further attempts to purify and concentrate the active factors involved.

O P J

**CYCLIC AGRANULOCYTOSIS** R. *Muratori* From the 2nd Medical Clinic Charles University, Prague Čas lék ces 86 1546, 1947

In a girl of 15 years suffering from agranulocytosis following immoderate use of amidopyrine (548 grams within one year) a marked recurrence of fever, leukopenia and appearance of necrotic areas in the gums could be observed in connection with the menstrual periods. Penicillin and transfusion were in effective but it was found that folic acid and pyridoxine were followed by a complete disappearance of all signs and symptoms.

M N

## THE SPLEEN

**SPLENOMEGALY** D. *Symmers* From the Laboratories of Pathology Bellevue Hospital New York City Arch Path 45 385-409 1948

This is a general review which, for convenience sake has assembled the splenomegalies into the following groups. Those of circulatory origin, mechanical, metabolic, blood dyscrasias, unknown nature and finally neoplastic and cystic splenomegalies. This excellent article has drawn upon material obtained from 23,792 necropsies at the Bellevue Hospital during the past 30 years.

O P J

**HAMARTOMA OF THE SPLEEN. REPORT OF THREE SURGICAL CASES** W. G. *Kirkland* and J. R. *McDonald* From the Division of Surgery Mayo Clinic Rochester Minn Arch Path 45 371-379 1948

Certain neoplasms consisting of an abnormal mixture of the normal components of an organ have been referred to as hamartomata ever since the term was proposed by Albrecht in 1904. Kirkland and McDonald studied splenic neoplasms removed surgically and found 3 cases which seemed to fall into this category. One of the outstanding features of each specimen was the ramifying spaces or channels lined by endothelium. It has been suggested that this is a specific benign tumor and that perhaps some hamartomata of the spleen previously reported actually belonged to this group.

O P J

**THE EFFECT OF HEAVY MUSCULAR WORK ON THE VOLUME OF CIRCULATING RED CORPUSCLES IN MAN** G. *Nylin* From Sabbatsberg's Hospital Stockholm Sweden Am J Physiol 149 180-4 1947

Since Barcroft's demonstrations in 1923-1925 that the spleen of dogs is capable of storing large quantities of blood for use in emergencies (exercise, administration of epinephrine), the reservoir function of the human spleen has been considered correspondingly well established. Little experimental verification of this thesis, however, has been offered.

In the present report Nylin tested whether severe muscular work could be shown to result in splenic contraction and emptying of the postulated stored blood in human adults. He injected blood containing labelled red blood cells (labelled with radioactive phosphorus) into 5 healthy men and took blood samples in ten and again in fifteen minutes after the injection. The subject was then made to do severe muscular work and two further blood samples were taken, the first at 25-32 minutes, the second at 27-39 minutes. All samples were subjected to radioactivity determinations in a Geiger-Müller counter. It had previously been shown by the author that the radioactivity of the blood remains constant for at least 60 minutes after such an injection; hence the volume of the circulating red cells could be measured by the radioactivity of the blood. Presumably, if any reservoir of blood was present which responded to the severe muscular exercise, discharge of red cells from this reservoir would change the radioactivity of the circulating blood.

Nylin found actually that there was no change in the specific activity of the blood after exercise within the time studies. For all the patients the mean circulating cell volume before work was 2,405 ml, as compared with 2,471 ml after work, and the mean circulating total blood volume before work was 4,934 ml, as compared with 4,855 ml after work. Since the amount of the red cells was unchanged, it

was concluded that there was no reservoir which empties red cells into the circulation after work. This result is in contrast with the work of Barcroft (on dogs), and with the commonly held opinions that epinephrine contracts the spleen and thereby increases the number of circulating red cells. If verified, these conclusions would be of great importance.

S.E.

## BLOOD COAGULATION AND HEMORRHAGIC DISEASES

MANAGEMENT OF GASTRIC HEMORRHAGE USING TOPICAL THROMBIN. T. M. Rogers. J. A. M. A. 137: 1035-1036, 1948.

This is a clinical report of the cessation of severe gastric hemorrhage in two cases following the introduction into the stomach of a solution of topical thrombin.

Especially impressive is the dramatic cessation of repeated massive, almost-exsanguinating hemorrhage in the first case: a 64-year-old man with a prepyloric ulcer. This patient bled repeatedly and severely despite rest, sedation (including the desperate use of pentothal sodium intravenously), epinephrine, vitamin K, parenteral feeding. As a final measure, 10,000 units of thrombin mixed with 2.5 cc. of isotonic sodium chloride solution were given orally, and repeated three times daily for five days. No bleeding occurred after the first dose of thrombin, and the patient progressively improved.

Although it is impossible definitely to demonstrate cause and effect in a case of this type, the clinical data strongly suggest that the thrombin was responsible for the cessation of gastric hemorrhage, and recommend its further trial in other similar cases.

S.E.

THROMBOCYTOPENIC PURPURA FOLLOWING QUINIDINE. P. L. Nadelman, I. L. Leff, and C. D. Howe. From the Third (New York University) Medical Division, Goldwater Memorial Hospital, New York, N. Y. J. A. M. A. 137: 1219-1220, 1948.

According to the authors, this is the second recorded instance of the development of thrombocytopenia following the use of quinidine. (Less rare is thrombocytopenia following quinine.)

The patient was a 37-year-old woman who received 0.6 grams of quinidine daily because of supraventricular tachycardia in hypertensive rheumatic heart disease. After she had taken 6.0 grams of the drug in 11 days, she began to bleed from the gums, and was found to have petechiae, ecchymoses, thrombocytopenia (4,000 platelets per cu mm), increased bleeding time, positive capillary fragility tests, and poor clot retractility. In the bone marrow, megakaryocytes were normal in number and appearance, and the differential count of the marrow cells was normal. A blood transfusion was given, and the patient made a rapid recovery. Subsequently, a test administration of 0.1 grams of quinidine resulted in an identical exacerbation of the syndrome.

S.E.

EXCESSIVE HYPOPROTHROMBINEMIA DUE TO DICUMAROL: ITS TREATMENT WITH LYOPHILIZED PLASMA. S. W. Coziff, R. J. Cross, and D. V. Hays. From the Departments of Medicine and Surgery, Columbia University College of Physicians and Surgeons, New York. J. A. M. A. 138: 405-6, 1948.

The authors suggest the feasibility of using reconstituted lyophilized plasma in the emergency treatment of excessive hypoprothrombinemia due to over-dicumarolization.

The prothrombin time of such plasma was found to be normal (12.7 to 15 seconds; control 12 to 16 seconds), and the administration of 500 cc. of such plasma to 13 patients with high prothrombin times (43.0 to 97 seconds) was found to cause a prompt return of the prothrombin time of the patients' plasma to safe levels (actually 22 to 38 seconds). The effect was transitory, however, and in some patients had disappeared by as little as six hours. Since none of the patients in the group had clinical bleeding, any effect on the hemorrhage of hypoprothrombinemia could not be ascertained.

The method may be added to the more conventional ones for emergency treatment of over-dicumarolization, viz., the use of synthetic vitamin K and the transfusion of whole blood. It would have been of interest to determine how small an amount of plasma suffices to shorten the prothrombin time.

S.E.

# NEWS AND VIEWS

## CONDENSATION OF THE FIRST TWO REPORTS OF THE COMMITTEE FOR CLARIFICATION OF THE NOMENCLATURE OF CELLS AND DISEASES OF THE BLOOD AND BLOOD-FORMING ORGANS\*

### THE TERMS AND DEFINITIONS FOR THE CELLS OF THE LEUKOCYTIC, THROMBOCYTIC AND ERYTHROCYTIC SERIES

Clarification and definition of terms is urgently needed for the sake of a common understanding in clinical usage and in teaching of medical students and technicians. The choice of a preferred term, it was agreed, should not be based merely on historical priority or common usage but, in general, should represent the simplest, clearest and most descriptive term. Eponyms and new terms should be avoided, wherever possible, without sacrifice of clarity. An effort should be made to attain consistency between related terms.

The various series of cells were considered. It was recommended that in table 1 the term listed at the left replace all terms listed at the right in referring to cells of a particular series or to a disease affecting any cell of that series.

No changes were suggested in the criteria in current use for determining the series to which a cell belongs. It is hoped, however, that the advances now being made in histochemistry will contribute more clearcut criteria than are available at present.

It is recommended that the term *leukocyte* be considered synonymous with white blood corpuscle and include all white cells of the blood and their precursors in the blood-forming organs. Its use should not be limited to cells of the granulocytic series, excluding cells of the lymphocytic, monocytic or plasmacytic series. This and other words derived from the same root should be spelled with a *k* and not a *c*: *leukocyte*, leukemia, not *leucocyte* or *leucemia*.

It is recommended that the descriptive terms for granulocytes: *neutrophil*, *eosinophil*, *basophil* and *azurophil* be spelled as indicated without a final *e*.

It is suggested that the name of the most undifferentiated of the cells of each series carry the suffix *blast*; the second stage the prefix *pro-* and except in the granulocytic series all cells that are more mature than the blast stage have names with the suffix *-cyte*. The name for the fourth stage in the granulocytic and erythrocytic series is to have the prefix *meta*. The terms *blast cells* and *pro cells* may be used to replace other terms for these stages of development when speaking of the stage of development as a whole or when the series to which the cells belong has not been identified.

It is recognized that the *blast cells* of each series are morphologically very similar, all having fine nuclear chromatin structure, usually demonstrable nucleoli and basophilic cytoplasm with or without azurophilic granules, so the prefix to be used will in many instances depend on the identification of the *pro-* stage associated with them.

*Fine chromatin structure* is defined as having the nuclear appearance of a background of homogeneous lighter staining parachromatin overlaid by a darker staining late net meshwork or finely stippled pattern.

\*Reprinted from the American Journal of Clinical Pathology 18 473 May 1948 and Vol 13 January 1949 with permission of the Editor and the publishers The Williams and Wilkins Company.

This condensation was made available by the Chairman of the Committee Dr. Edwin E. O'Rand, Portland, Oregon.

tern of basichromatin with no aggregation of the basichromatin into even a single clump of appreciable size staining darker than any other areas in the nucleus

A *nucleolus* is defined as a homogeneous blue staining area within the nucleus of a cell, which stains more like the cytoplasm than does any other part of the nucleus

The term *azurophil* should be applied to the granules seen typically in the cytoplasm of cells of the lymphocytic and monocytic series and the progranulocyte stage of the granulocytic series. The term *azurophil* is recommended, and not *azuric*, in describing these granules, since the term refers to an affinity for a particular dye and not to the color of the granules. These granules may be present or absent in any cell of the lymphocytic series and when present are usually coarse and in clumps. They are usually present in all cells of the monocytic series, including the monoblast. In the monocytic series they are usually fine, diffusely and uniformly scattered through the cytoplasm. If not seen in the monocyte or promonocyte, it usually indicates a faulty stain or poor visual definition in the microscope. These granules may be present or absent in any cell of the granulocytic series. They are rarely seen beyond the myelocyte stage except in disease. They are occasionally present in the cytoplasm of cells of the plasmacytic and erythrocytic series and constantly present in the cells beyond the blast stage in the thrombocytic series where they tend to be fine and few in the early stages and numerous and often clumped in the more mature stages.

TABLE 1 — *Recommended Terms and Terms to be Avoided when Referring to Cells of a Particular Series in a Disease Affecting any Cell of that Series*

Term to be used	Terms to be avoided
Lymphocytic	Lymphoid lymphatic, lymphogenous, lymphocyte, mononuclear
Granulocytic	Myeloid myelogenous, myelocyte, myelocytic, granulocyte, leukocyte leukocytic, leucocyte, leucocytic
Monocytic	Monocytoid monocytogenous, mononuclear, monocyte
Plasmacytic	Plasma cellular plasmacytogenous myeloma cell plasmacyte
Thrombocytic	Megakaryocytic platelet, thrombocyte
Erythrocytic	Erythroid erythrocytoid erythron, erythrocytogenous, erythrocyte

It is recognized that in each cell series there is a continuous development from the most undifferentiated to the most differentiated stage, that an infinite number of subdivisions are possible, and that any subdivision is arbitrary. The committee recommended the use of the minimum number of subdivisions which will provide essential information for diagnostic and prognostic purposes and defined the lines of division between these stages as clearly as possible, basing these divisions on a single easily identifiable feature. As far as possible, the feature selected to differentiate the different stages of development is one which could be recognized in either stained or supravital preparations, but it is realized that at present the majority of such decisions will be based on smears stained with Wright's stain or with one of the other Romanowsky stains. Even with these definitions, cells will be encountered where decision is difficult, in which case it is suggested that the cell be arbitrarily placed in the more differentiated category.

Names were selected for each of the cells, which were acceptable to all members present and which, in the opinion of the committee, were least likely to be confusing.

The recommended terms and the terms to be avoided are listed in table 2.

*It is not the intention of the committee to imply from its recommendation of terms to be used that the origin of all these cells has been settled.*

It is recognized that to ensure flexibility and for certain specialized purposes finer

TABLE 2.—*Recommended Terms and Terms to be Avoided when Referring to Specific Cells of the Blood and Blood Forming Organs*

<i>Name of series</i>	<i>Term to be used</i>	<i>Terms to be avoided</i>
Lymphocytic	Lymphoblast	Myeloblast, hemocytoblast, lymphoidocyte stem cell, lymphocyte
	Prolymphocyte	Large lymphocyte pathologic large lymphocyte atypical leukocytotic lymphocyte monocyte immature lymphocyte
	Lymphocyte	Small medium or large lymphocyte, normal lymphocyte, small, medium or large mononuclear
Monocytic	Monoblast	Myeloblast hemocytoblast, lymphoidocyte lymphocyte, stem cell, immature monocyte
	Promonocyte	Premonocyte, hemohistioblast immature monocyte Ferrata cell
	Monocyte	Large mononuclear transitional, plasmacyte endothelial leukocyte histiocyte resting wandering cell
Granulocytic	Myeloblast	Granuloblast hemocytoblast, lymphoidocyte lymphocyte stem cell
	Progranulocyte	Promyelocyte II leukoblast myeloblast promyelocyte promyelocyte progranulocyte A
	Myelocyte	Granulocyte, myelocyte B, non filament class I
	Metamyelocyte	Metagranulocyte juvenile, myelocyte C, non filament class I
	Band Cell	Staff cell stab cell non filament, class I rod nuclear polymorphonuclear stabkernige, rhabdocyte non segmented
	Segmented	Polymorphonuclear filamented class II, III, IV or V lobocyte
Plasmacytic	Plasmablast	Myeloblast, hemocytoblast, lymphoidocyte lymphocyte, stem cell, lymphoblastic plasma cell myeloma cell
	Proplasmacyte	Türk cell, Türk irritation form lymphoblastic or myeloblastic plasma cell myeloma cell
	Plasmacyte	Plasma cell Unna's plasma cell, Marschalko's plasma cell plasmacytotic lymphocyte myeloma cell
Thrombocytic	Megakaryoblast	Megalokaryoblast
	Promegakaryocyte	Premegalokaryocyte
	Megakaryocyte	Megalokaryocyte
	Thrombocyte	Platelet thromboplasmic
	Disintegrated cell	Senile cell smudge basket cell smear cell dgerat dec



subdivisions may be necessary than those herein recommended. It is suggested that in such case no change be made in the term or definition of the recommended major divisions but that *clearly defined* qualifying adjectives be used for these further subdivisions. Should new knowledge indicate that another major cell division is needed the evidence for this need, together with the suggested term, should be submitted for consideration by a permanent body which it is hoped will develop out of this committee.

The definitions decided on are as follows:

*Lymphoblast* Any cell of the lymphocytic series having fine chromatin structure in the nucleus. Cells of blast morphology associated with prolymphocytes should be tentatively classified as lymphoblasts.

*Prolymphocyte* Any cell of the lymphocytic series intermediate in morphology between the lymphoblast and the lymphocyte. It will always have too coarse a chromatin structure to fit the criteria for a blast and too fine a chromatin structure or too large a cell diameter to be classed as a lymphocyte. Usually but not always prolymphocytes are larger than 15 microns in diameter which is the upper limit for the lymphocyte.

*Lymphocyte* Any cell of the lymphocytic series having the morphology of those commonly found in the blood of healthy adults.

*Monoblast* Any cell of the monocytic series having fine chromatin structure. Usually nucleoli are visible. Cells of blast morphology found in association with promonocytes should be tentatively classed as monoblasts.

*Promonocyte* Any cell intermediate in morphology between the monoblast and the monocyte. It is differentiated from the monoblast by having an irregularly shaped nucleus and somewhat coarser chromatin structure and from the monocyte by the presence of one or more nucleoli.

*Monocyte* Any cell of the monocytic series having the morphology of those commonly found in the blood of healthy adults. It is differentiated from the promonocyte by the absence of nucleoli.

*Myeloblast* Any cell of the granulocytic series having fine chromatin structure and containing no specific granules. Usually nucleoli are visible. Cells of blast morphology found in association with progranulocytes should tentatively be classed as myeloblasts.

*Progranulocyte* Any cell of the granulocytic series which has a nuclear structure too coarse for that of a blast cell and which has not yet developed discernible specific granules. This term was selected rather than promyelocyte because of its clear relationship to the definition of granulocyte, given below, and because the term promyelocyte has been in wide use for cells which do contain specific granules. The reason that the terms granuloblast, granulocyte and metagranulocyte were not chosen was that the terms myeloblast and myelocyte were already in general use with essentially the definitions here given. This is true also for the term granulocyte which would otherwise have to be synonymous with the term myelocyte.

*Specific granules* Neutrophilic, eosinophilic or basophilic granules. This term does not include *azurophilic* granules.

*Granulocyte* An inclusive term to apply to any cell containing specific granules. The plural form *granulocytes* would therefore include all myelocytes, metamyelocytes, band cells and segmented cells whether neutrophils, eosinophils or basophils.

*Myelocyte* Any cell containing specific granules, with a round or oval nucleus. It is distinguished from the progranulocyte by the presence of specific granules and from the metamyelocyte by the absence of indentation in the nucleus. It may be further subdivided at the option of the user into early and late stages but the definition of early or late should be clearly stated in any publication.

This and all subsequent cells of the granulocytic series should be additionally characterized as neutrophil, eosinophil or basophil.

*Metamyelocyte* Any cell of the granulocytic series having specific granules in the cytoplasm and a nucleus intermediate in shape between that of the myelocyte and the band cell. The nucleus usually has an indented oval shape, resembling a bean or kidney.

**Band cell** Any cell of the granulocytic series which has a nucleus that could be described as a curved or coiled band, no matter how marked the indentation, if it does not completely segment the nucleus into lobes connected by a filament. It is differentiated from the metamyelocyte by an appreciable length of the nucleus having parallel sides, and from the segmented neutrophil by having no indentation which could be described as a filament.

**Segmented cell** Any cell containing specific granules in which the lobes of the nucleus are connected by a filament. A *filament* is defined as a threadlike structure. Since at times in viewing a three-dimensional object from one direction, it is impossible to be certain whether two parts of the nucleus are connected by a filament or band, it is suggested that such cells always be placed in the segmented category, since this is the more differentiated and more common cell.

The term *toxic neutrophils* followed by a 1 to 4+ designation is recommended for the grading of toxic granules, basophilia of the cytoplasm, vacuoles and condensation of nuclear chromatin in the neutrophils, since its meaning is clear although it is recognized that it is not an adequately descriptive term. The grading should depend more on the degree of change than on the percentage of the cells involved and should be recorded in the report whenever the degree of change exceeds 2+.

**Plasmablast** Any cell of the plasmacytic series having fine chromatin structure in the nucleus. Cells of blast morphology found in association with proplasmacytes are usually seen only in plasmacytic leukemia or plasmacytic sarcoma. The cytoplasm tends to be more opaque in staining than in the other leukocytic blast cells.

**Proplasmacyte** Any cell of the plasmacytic series with a nuclear structure too coarse for that of a blast cell but with one or more nucleoli present.

**Plasmacyte** A cell characterized by extremely coarse chromatin structure with the deeply staining chromatin of the nucleus aggregated into large sharply demarcated clumps. It is differentiated from the proplasmacyte by the absence of nucleoli. The cytoplasm of all cells of the plasmacytic series tends to be deeply basophilic and opaque in appearance. Azurophilic granules may be present or absent but are more commonly absent.

**Megakaryoblast** Any cell of the thrombocytic series having a nucleus with fine chromatin structure. Usually these are larger than the other blast cells.

**Pronegkaryocyte** Any cell of the thrombocytic series with a nucleus containing nucleoli but having a chromatin structure too coarse for a blast cell. The nucleus is usually similar in shape to that of the megakaryocyte. Fine azurophilic granules are usually diffusely scattered through the cytoplasm.

**Megakaryocyte** Any nucleated cell of the thrombocytic series in which nucleoli are not discernible. The azurophilic granules are often aggregated into clumps. Megakaryocytes and pronegkaryocytes are typically much larger than other cells found in the marrow.

**Thrombocyte** Any cell of the thrombocytic series containing no nucleus in other words any non nucleated fragment of megakaryocytic cytoplasm containing azurophilic granules similar to those of the mature megakaryocyte.

The term *thromboplastid* was recognized as being anatomically correct but it was felt that to be consistent with the use of the term *erythrocyte* and to permit the use of thrombocytic and erythrocytic in describing these cell series the suffix *-cyte* was preferable for these two non nucleated forms.

**Disintegrated cell** Any cell of any series in which the cytoplasmic outline has been disrupted or the nuclear chromatin is no longer surrounded by a membrane excluding the changes in the nucleus that occur in mitotic division. Disintegrated cells should be recorded as such in the differential report even though they could be identified by dispersed granules. They should be counted even if only shreds of nuclear material are discernible since they are undoubtedly included in the total leukocyte count.

It was the decision of the committee that none of the terms in current use for the nucleated cells of the erythrocytic series could be recommended because mutually exclusive definitions for the same term have been used in different schools of hematology, because these are all inconsistent with the terms already recommended by the committee for the other series of cells, and because the use of the suffix *-blast* for the most differentiated nucleated cell of the erythrocytic series has been a constant source of confusion to medical students and medical technologists for in

all other series -blast has been used exclusively for the least differentiated cell. The logical terms *erythroblast*, *proerythrocyte*, *erythrocyte* and *metaerythrocyte* were impossible to use because of the wide employment of the terms *erythroblast* and *erythrocyte* with other meanings than would be intended for them in the present recommendations. After considering many suggestions and consulting Latin and Greek authorities, the Latin syllables *rubri*, meaning red, were selected as least likely to be misinterpreted because this stem is familiar in medical terminology, having been used in *polycythemia rubra vera* and in the derivation of many other words in which the root *rub* denotes red, such as *rubicund* and *rubefacient*. Other stems considered were the Greek terms *roso*, rose, *rosino*, rosy, *erythe*, red, *porphyro*, deep-red, *pyrrho*, flame-colored, and *cirrho*, tawny-yellow, but these were discarded as likely to be more difficult to pronounce and learn.

TABLE 3 — Recommended Terms and Terms to be Avoided when Referring to Specific Cells of the Erythrocytic Series

Name of series	Term to be used	Terms to be avoided
Erythrocytic	Rubriblast	Erythroblast megaloblast pronormoblast promegaloblast, normoblast, hemocytoblast stem cell myeloblast lymphoidocyte karyoblast
	Prorubricyte	Erythroblast megaloblast pronormoblast normoblast macro-normoblast macroblast prokaryocyte
	Rubricyte	Normoblast pronormoblast macronormoblast erythroblast polychromatophilic normoblast karyocyte
	Metarubricyte	Normoblast erythroblast metakaryocyte
	Reticulocyte*	
	Erythrocyte	Red blood cell erythroplastid normocyte, akaryocyte

\* It is recommended that the reticulocyte stage be considered a subdivision of the erythrocyte stage.

The best solution that could be found for the problem of clearly indicating the changes in nuclear morphology commonly seen in cells of the erythrocytic and granulocytic series in pernicious anemia and other macrocytic anemias which respond to liver extract and folic acid was to coin a new adjective phrase which could be used to qualify the recommended terms for any of the cells of these two series, or to describe the marrow and blood pictures as a whole. The terms *macrocytoid*, *macroid*, *megalo-*, and *megaloid* were considered, but none was acceptable to the authorities consulted or to the majority of the members of the committee. The adjective phrase *pernicious anemia type* was recommended by the committee after extensive deliberation, to be used in full in any publication, although in the laboratory and clinic it can conveniently be abbreviated to *P A*. The use of such an adjective phrase should be perfectly clear and it has the great advantage over *megalo-blastic* that it can be applied to cells of the granulocytic as well as of the

erythrocytic series and also to the marrow and blood pictures. Eventually, if the anti-pernicious anemia principle is identified and given a short, simple name, a term analogous to *afolic* may be substituted by committee action for the presently recommended adjective phrase.

The names selected by the committee for the stages of differentiation are given in table 3, and their definitions follow. It should be re-emphasized, as was pointed out in the first report,<sup>1</sup> that no changes are suggested or implied by these definitions for the criteria in current use for determining the series to which a cell belongs. The recommended definitions are meant to point out only the one essential differential characteristic for determining the stage of differentiation, and they are not intended to be complete descriptions of all cell stages, or of normal and pathologic variants. For these finer details of identification readers are referred to standard textbooks of hematology.

**Rubriblast.** Any cell of the erythrocytic series having fine chromatin structure in the nucleus. Nucleoli are usually discernible. A stippled chromatin pattern is more common than the lace-net pattern usually seen in other blast cells.

**Prorubricyte.** Any cell of the erythrocytic series in which one or more nucleoli are discernible in the nucleus and which has a chromatin structure too coarse to be classified as a rubriblast.

**Rubricyte.** Any cell of the erythrocytic series having definite structure of the nuclear chromatin, but containing no discernible nucleoli. This stage is differentiated from the prorubricyte by the absence of nucleoli in the nucleus and from the metarubricyte by not having a pyknotic, fragmented or partially extruded nucleus. Some may wish to subdivide and qualify this stage—or other stages—further into basophilic, polychromatic or normochromatic rubricytes according to the amount of hemoglobin present in the cytoplasm.

**Metarubricyte.** Any nucleated cell of the erythrocytic series having a pyknotic, fragmented, partially extruded or partially autolyzed nucleus. *Pyknotic* describes a dense, solid, structureless nuclear mass. The phenomenon of karyorrhexis or fragmentation of nuclei should be clearly distinguished from the occurrence of double, well formed nuclei which are occasionally seen in prorubricytes and rubricytes as well as in other cells which may divide amitotically.

**Reticulocyte.** Any non nucleated cell of the erythrocytic series in which, when supravitality stained—usually with brilliant cresyl blue—one or more granules or a diffuse network of fibrils are discernible. All reticulocytes are included under the term erythrocytes since, without a special stain, reticulocytes are indistinguishable from erythrocytes.

**Erythrocyte.** Any non nucleated cell of the erythrocytic series.

**Pernicious anemia type.** The qualifying adjective phrase to be applied to any cell of the erythrocytic or granulocytic series and to the marrow and blood pictures as a whole to indicate the presence of the morphologic changes characteristically seen in pernicious anemia and other macrocytic anemias which respond to liver extract or folic acid therapy. In the nucleated cells of the erythrocytic series the major feature of this change is a relative increase in the pale staining parachromatin with a corresponding decrease in the deep-staining basichromatin. In the cells of the granulocytic series the characteristic change is the presence of giant forms having very bizarre nuclei and in the segmented neutrophils the occurrence of many cells with more than five lobes.

Each name recommended for the cells of the erythrocytic series clearly indicates the stage of differentiation. The use of the qualifying adjective phrase, *pernicious anemia type*, with the name of the cell stage will equally clearly indicate that a cell shows the alterations in morphology typically seen in the marrow or blood of untreated pernicious anemia, as contrasted with the corresponding cell which is unqualified as to terminology. Pre-existing confusion in the usage of terms for

nucleated erythrocytes is thought to be clarified by the recommended terminology as illustrated by the following example *Megaloblast* as in current use by some hematologists is synonymous with *rubriblast*, as herein recommended and defined, but as used by other hematologists it is synonymous with the presently recommended term *pernicious anemia type prorubricyte*

It is, of course, understood that modifying adjectives may be applied to any of the recommended terms in describing results of investigation, but if these terms are to gain general acceptance they should not be given any new definitions except by general action of the committee

#### REFERENCES

- <sup>1</sup> First report of the Committee for Clarification of the Nomenclature of Cells and Diseases of the Blood and Blood Forming Organs *Am J Clin Path* 18 443-450, May 1948
- <sup>2</sup> Second report of the Committee for Clarification of the Nomenclature of Cells and Diseases of the Blood and Blood Forming Organs *Am J Clin Path* Vol 19 January, 1949

#### INTERNATIONAL SOCIETY OF HEMATOLOGY

Through an unfortunate error in publication, the names of the two Secretaries-General were omitted from the list of officers elected at the Buffalo Congress of last August (*Blood* 3 1313, 1948). The complete list of new officers is as follows

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A more complete report of the Congress, of which the November 1948 note was intended as preliminary, will be published in a future issue of *Blood* as soon as the large amount of wire-recorded material can be transcribed and edited. The publication of a volume of the Proceedings is presently being discussed

Acting on suggestions made to the Editorial Board, the Journal is pleased to announce the following revision in subscription policy. Holders of Internships, Residents and Fellowships within the United States may now obtain one or two year subscriptions to *Blood* at the reduced rate of \$9 per year. Those wishing to take advantage of this offer should provide the publisher with their hospital addresses and positions

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## *Contributors to this Issue*

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DAVID H APPLEMAN, M D

Adj. in Medicine Beth El Hospital, Brooklyn,  
N Y

J H BOLTON, M D

Royal Melbourne Hospital, Melbourne, Victoria,  
Australia

WILLIAM DAMESHEK, M D

Hematologist J H Pratt Diagnostic Hospital,  
Professor of Clinical Medicine Tufts College  
Medical School Boston Mass

CHARLES S DAVIDSON, M D

Associate in Medicine Harvard Medical School  
Associate Physician Thorndike Memorial Labora-  
tory, Junior Visiting Physician Boston City  
Hospital Boston Mass

D G DIETER, M D

Department of Physiology University of Wiscon-  
sin Medical School now associated with Mad-  
ison General Hospital Madison, Wis

ROBERT D EPSTEIN, M D

Assistant in Medicine Harvard Medical School,  
Research Fellow Thorndike Memorial Labora-  
tory, Assistant in Medicine Boston City Hospital  
Boston Mass

SIDNEY FARBER, M D

Department of Pathology, Harvard Medical  
School, The Children's Medical Center Boston  
Mass

LUTHER W GRAY, M D

Member, American Board of Surgery Surgical  
Facility, Georgetown University School of Med-  
icine, attending staffs of Emergency, Providence,  
Doctors Georgetown University and Gallinger  
Municipal Hospitals, Washington, D C

GEORGE F MILLER, M D

Instructor in Surgery Boston University School of  
Medicine Assistant to Visiting Surgeon, Boston  
City Hospital, Boston Mass

CLARENCE A MILLS, M D, Ph D

Department of Experimental Medicine, University  
of Cincinnati, Cincinnati, Ohio

MAURICE M MORRISON, M D

Att Hematology, Beth El Hospital Att Hema-  
tology, Jewish Hospital Brooklyn, N Y

HERBERT M PERR, M D

Resident in Medicine Veterans Administration  
Hospital, Bronx, N Y

CLAUS MUNK PLUM, Ph D

Department of Pathological Anatomy University  
of Copenhagen, Copenhagen, Denmark

F J POHLE, M D

Deceased Formerly Associate Professor of Medi-  
cine University of Wisconsin Medical School,  
Madison Wis

KARL ROHR, M D

Medical Department University of Zurich Zu-  
rich, Switzerland

M SPOONER, M A

Graduate Assistant Department of Physiology,  
University of Wisconsin Medical School Madison  
Wis

F H L TAYLOR, Ph D

Associate in Research Medicine, Harvard Medical  
School, Chemist Thorndike Memorial Labora-  
tory Director Biochemical Laboratories, Boston  
City Hospital, Boston, Mass

CHARLES E WARD, M D

Fellow American Academy Pediatrics, private  
practice Jackson, Miss, formerly Major, M C  
A U S

SLOAN J WILSON M D

Hematologist University of Kansas Medical  
Center Assistant Professor of Medicine Univer-  
sity of Kansas School of Medicine Kansas City,  
Kansas

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# BLOOD

## *The Journal of Hematology*

VOL IV, NO 2

FEBRUARY, 1949

*The first six articles in this issue conclude the George Minot Anniversary Volume*

### HEMOPHILIA A CLINICAL STUDY OF FORTY PATIENTS

By CHARLES S. DAVIDSON, M D, ROBERT D. EPSTEIN, M D, GEORGE F. MILLER, M D, AND F. H. L. TAYLOR, PH D

#### INTRODUCTION

SINCE 1803 when hemophilia was first accurately described by Dr John C Otto,<sup>1-3</sup> investigators of this disease have centered their efforts chiefly in the elucidation of its hereditary nature and in the study of the constant defect in blood coagulation. In the study presented here we wish to emphasize certain clinical manifestations of the disease and methods of practical therapeutic management which have been learned in this laboratory during the last ten years in the course of a study of the defect in coagulation of the blood in individuals with the disease. The deep interest of Dr George R. Minot in hemophilia began in 1918 when he and Dr Roger I. Lee first demonstrated in this country that whole blood transfusions were effective in shortening the blood coagulation time.<sup>4</sup> He has been the guiding spirit of the investigative work in hemophilia in this laboratory, and this presentation is dedicated to him. His guidance in this problem has given experience to many young men in the methods of clinical investigation.

Hemophilia is an hereditary disease limited to males, those afflicted exhibiting both impaired coagulability of the blood and a strong tendency to bleed especially following trauma. Although there may be variations in the frequency and severity of hemorrhagic episodes, the disease is always present for life. Transmission of the disease is always through the female to the second generation, the genes being sex-linked and recessive. Although the possibilities exist of both a first generation male with hemophilia as well as a female with the disease, authenticated cases are not known.

Although Otto was the first to bring the true nature of the disease into clear focus, there is evidence that certain aspects had been known in ancient times by the Arabs and the Jews. Bullock and Fildes<sup>5</sup> in their classical monograph on the disease report descriptions of a condition resembling hemophilia by Albucasis in the tenth century. Among the recent general articles or monographs on hemophilia are those of Birch,<sup>6</sup> Howell,<sup>7</sup> Stetson and Lozner,<sup>8</sup> Quick,<sup>9</sup> Davidson and McQuarrie,<sup>10</sup> Ely,<sup>11</sup> Mills,<sup>12</sup> MacFarlane,<sup>13</sup> and Kark.<sup>14</sup>

From the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard) and the Department of Medicine, Harvard Medical School, Boston, Mass.

This study was aided in part by a grant from the Smith Kline and French Laboratories, Philadelphia, Pa.



The coagulation defect in hemophilia is observed *in vitro* as a prolongation of the whole blood clotting time. Normal blood clots in from 6 to 12 minutes as measured by a modification<sup>15</sup> of the method of Lee and White.<sup>16</sup> The blood of a patient with hemophilia under the same circumstances may not clot for many hours. However, most of the patients in our series have clotting times of from 1 to 2 hours, but in a few the clotting time is in the range of 20 to 40 minutes. Although it has been reported<sup>6</sup> that patients with hemophilia will occasionally exhibit a normal coagulation time, this phenomenon has never been observed in this laboratory.

The coagulation defect in hemophilia has been ascribed by some to an abnormality of the platelets<sup>4</sup> and by others to the presence of an antithrombin.<sup>17</sup> However, hemophilic blood will coagulate promptly upon the addition of thrombin. Tocantins has described the presence of an antithromboplastin in hemophilic blood,<sup>19</sup> while workers in this laboratory believe that there is a deficiency of some factor associated with plasma globulins.<sup>18</sup> Nevertheless, it is generally agreed that fibrinogen, prothrombin, calcium, and the *number* of platelets are all normal in hemophilic blood. Thus, whatever the abnormality, for practical purposes transfusion of whole blood or plasma and certain plasma derivatives will usually bring the blood coagulation time to or near to normal. Hemophiliacs have been observed<sup>20-22</sup> whose coagulation time does not respond as is customary to the administration of blood, plasma or its derivatives. The basis for this failure to react has not been fully elucidated.

Fractionation of blood plasma has led to the identification of the antihemophilic activity with the  $\gamma$ -globulins<sup>16, 23, 24, 25</sup> and particularly with fraction I,<sup>26</sup> according to the nomenclature used by Cohn et al., Fraction III-2 also contains considerable antihemophilic activity. Because of the impracticability at present of the administration of fraction III-2, the fibrinogen fraction I has been chiefly studied *in vivo*<sup>27</sup> and contains antihemophilic activity which has been clearly shown not to be fibrinogen itself.<sup>28, 29</sup>

Occasionally patients are seen who have suffered hemorrhagic episodes but whose coagulation time is only slightly prolonged. It is very difficult either to establish or exclude the diagnosis of hemophilia in these patients, particularly if a family history of the disease is not obtained, as is frequently the case. Certain laboratory procedures may be helpful in this regard. Among these is the well-established reduction in the clotting time of hemophilic blood by the addition of small amounts of normal plasma or its derivatives.<sup>28</sup> The measurement of the recalcification time of plasma centrifuged at different speeds<sup>31</sup> may prove to be a valuable diagnostic test, if substantiated.

#### CLINICAL MANIFESTATIONS OF HEMOPHILIA

It is our purpose to report observations on the clinical manifestations and practical management of forty patients with hemophilia, all of 12 years of age or over, who have been followed in the Thorndike Memorial Laboratory during the last ten years. All were males, the youngest 12, the oldest 58. Eight were in the second

decade of life, 19 in the third, 7 in the fourth, 5 in the fifth, and one in the sixth

Twenty-eight of the 40 (70 per cent of the series) had a family history of hemophilia. Twenty-five had a known member of the family in the same generation with the disease, 14 one generation back, 4 two generations, while none was able to trace the disease further. The lack of a positive family history is in part due to inadequate knowledge on the part of the patients about their families.

There were three patients in whom the family history was known and in whose family no hemophilia had appeared during three previous generations. Whether these instances represent sporadic hemophilia or whether the disease was carried by the female through successive generations without manifestation in a male offspring is not known, but the latter possibility would appear to have more support from the literature.

Spontaneous hemophilia has been reported, the most recent article by Boggs<sup>32</sup> reviews the reported cases and presents six brothers with the disease whose family history gave no evidence of bleeders, although it was known for four generations on the mother's side. Boggs admits that the legitimacy of the mother could be questioned. The statistical likelihood of the occurrence of hemophilia and of carriers in families has been studied by Haldane and Philip<sup>33</sup> who have said: "the daughters of hemophilic men bear equal numbers of normal and hemophilic sons, whilst half the sisters of hemophilic men are heterozygous for hemophilia." The number of individuals in the two sexes in hemophilia was shown to be normal by Macklin.<sup>34</sup>

Most of our cases were of recent European extraction. The family extractions (known in 38 of the families) were: New England 6, Nova Scotia 7, Irish 8, Italian 7, Jewish 3, English 2, Eastern European 5. There were no Orientals or Negroes in the present series, although hemophilia has been reported in both mixed and presumably full-blooded Negroes<sup>35, 36, 37, 38</sup> and six probably authentic cases have been described in native Japanese.<sup>39</sup> Ten of the patients in this series are married with a total of 13 children, 3 males and 10 females. There are no grandchildren.

There were five deaths in the series of forty patients in ten years. Three of these were from conditions quite unrelated to hemophilia, one, age 16, from fractured skull and broken leg following an automobile accident, the second, age 32, from cerebral hemorrhage in terminal malignant hypertension, and the third, age 34, from pulmonary tuberculosis. The fourth, age 21, developed an apparently spontaneous massive hematoma in the left gluteal and thigh muscles with secondary necrosis, slough and sepsis. The fifth death was from rapid submucosal pharyngeal and laryngeal hematoma formation which blocked the airway before help was available.

There were no deaths in this series from acute blood loss, the popularly supposed cause of death in hemophilia. This was in spite of frequent tooth extractions and five relatively serious operative procedures (cf. section on Treatment, Surgery in Hemophilia). Moreover, most of the patients at some time have been admitted to the hospital with a severe hemorrhagic episode.

### *First Hemorrhagic Episode*

In 36 of the patients the first hemorrhagic episode was known and varied in time of onset from the age of one week to 13 years. Three were following circumcision in the first two months (two at the age of one week). Eight others had their first bleeding during the first year of life, two developed an hematoma of the head from known trauma, two bled from cut lips, one had an hemarthrosis of the knee, one hematoma around the knees from crawling, one multiple hematomata, and for one the precise nature of the bleeding had been forgotten. The remaining 25 patients experienced their first hemorrhagic episode during childhood, 19 before five years of age having a variety of hemorrhagic lesions not differing essentially from those to be described for adult life and in most following known trauma.

Excessive bleeding from primary dentition occurred in only one instance of the 22 in whom the history was available, while 13 of 22 had excessive bleeding from secondary dentition. Hemorrhage following the extraction of permanent teeth is much more frequent and will be discussed in a separate section.

### *Hemarthrosis*

Bleeding into joints is the most frequent hemorrhagic episode in adult hemophiliacs. It is usually repeated often so that eventually many joints acquire some degree of permanent damage. Thirty-six of the patients in this series had chronic hemophilic joint disease and almost all of these gave a history of one or more acute hemarthroses. Of the four who exhibited no chronic joint disease and had no history of acute hemarthroses, two had suffered relatively few hemorrhagic episodes of any kind.

Acute hemarthroses and chronic hemophilic joint disease affected the joints in about the same incidence, the knees and elbows being by far the most frequently involved. The ankles, hips and shoulders were affected much less frequently, and the wrists, fingers and toes only occasionally.

Acute hemarthroses frequently occur without known external trauma, although joints, especially those bearing weight, are subject to the continual trauma of movement. The hemarthrosis is heralded by stiffness that soon becomes painful on movement of the joint. It is followed within a few hours by swelling which gradually distends the joint capsule causing severe pain even at rest, being greatly aggravated by motion. Tenderness is exquisite and limited, at least at first, as is the swelling, to the areas where the joint surface is relatively superficial. For example, in the elbows, the areas lateral to the olecranon are swollen, tense and exquisitely tender. The blood may break through the tense capsule and be released into the neighboring tissues, temporarily relieving somewhat the pain and tenderness of the hemarthrosis. When this occurs the blood may dissect superficially giving the typical discoloration of an ecchymosis. However, usually the blood remains confined to the joint and discoloration is then not observed. It is because of this lack of discoloration around a joint that an acute hemarthrosis is sometimes mistaken for other forms of acute arthritis. During the acute phase the joint is usually held in the position of greatest relaxation, the knees and elbows, for

example, in partial flexion, and any attempt to change the position is attended by severe pain

Usually in from four to six days recovery from the acute phase begins. Pain and tenderness subside a little and the previously tense stretched skin over the joint shows a fine wrinkling. Recovery usually then proceeds rapidly but may require two or three weeks before it is maximal. Residual limitation of motion is common and may become permanent, particularly if the joint has been the object of frequent previous attacks.

Acute hemarthrosis has been mistaken for acute rheumatic fever, rheumatoid, gonococcal and other types of arthritis, but may be readily differentiated if hemophilia is considered.

### *Chronic Hemophilic Joint Disease*

Following repeated acute hemarthroses a chronic and often deforming joint disturbance occurs. This is not to be thought of as a chronic hemarthrosis, but rather as the result of frequent irritation to the joint leading to roughening of the joint surfaces and fibrosis together with both areas of bone reabsorption and new bone formation. The description of both acute and chronic hemophilic joint disease by König<sup>40</sup> is the classical one, but a considerable body of literature has been published on the subject. Caffey and Schlesinger<sup>41</sup> point out that coxa plana resembling Perthes disease may be the result of joint hemorrhage and further that epiphyseal overgrowth and precocious ossification may be demonstrated by x-ray. Fonio,<sup>42</sup> Newcomer,<sup>43</sup> Lamv,<sup>44</sup> Keifer and Myers,<sup>45</sup> and MacDonald and Lozner,<sup>46</sup> have discussed the clinical and x-ray findings. The latter two papers are based on patients included in the series reported here.

In spite of active preventive measures, such chronically affected joints usually show some limitation of motion and may eventually become ankylosed. The joints are enlarged, the characteristic fusiform appearance being accentuated by atrophy of muscles on either side of the joint. Tenderness and pain on movement are not characteristic of chronic hemophilic arthritis, in fact, if either is present, recent active bleeding has probably occurred.

In only two of this series of forty hemophiliacs was there no evidence of chronic hemophilic arthritis. One would, in fact, hesitate to make the diagnosis of hemophilia without the presence of joint deformity unless the diagnosis could be otherwise conclusively established.

Arising usually after extensive bleeding into and around a joint, Volkmann's contracture sometimes becomes a serious deformity, greatly limiting usefulness of the extremity.<sup>47-50</sup>

### *Hemorrhage into the Skin, Subcutaneous Tissue and Muscles*

Purpura is not the characteristic phenomenon in hemophilia that it is in purpura hemorrhagica. Ecchymosis and hematoma when they occur usually follow known trauma rather than appear spontaneously as they do in purpura hemorrhagica. Ecchymoses seldom spread extensively but hematomata into subcutaneous tissue often spread until they are limited by fascial attachments.

Bleeding into muscle almost always follows severe trauma and may spread rapidly, usually into the subcutaneous tissue and along fascial planes. Subcutaneous and intramuscular hematomata are usually much larger than superficial examination would suggest. Shock from blood loss is not uncommon, and anemia, icterus (with an increased indirect serum van den Bergh reaction), reticulocytosis and urobilinogenuria follow. Hemorrhage into the gluteal region with spread into the thigh is one of the most common and because of the amount of available space may be extensive and lead to early shock.

### *Hemophilic Pseudo Tumor*

Occasionally, bleeding into or around bone tissue may be extensive and persistent enough to interfere with the blood supply and cause reabsorption of bone. This is observed chiefly in the hands or feet and the part may be converted in the course of weeks or months into a swollen, tense sac of old blood and destroyed tissue. X-ray examination is usually misinterpreted as sarcoma because of the soft tissue swelling and bone absorption. Firor and Woodhall<sup>51</sup> reviewed the literature on this subject and reported a case of their own, a 15-year old boy who developed a gradually progressive swelling of the right thumb over 18 months following injury. X-ray revealed absorption of bone and a diagnosis of bone sarcoma was made. Successful amputation was done with the aid of an electric cautery. A 16-year old boy in our series had a similar occurrence which developed over the course of almost a year and involved the left foot from the mid-tarsus distally. The metatarsal bones were almost completely resorbed and an x-ray diagnosis of sarcoma was made. Surgical amputation was done with great care and with a good result.

In addition to the pseudo tumor of the distal end of the extremities, other changes such as calcification in a subperiosteal hematoma have been described as sarcoma. In these instances there may be reabsorption of bone also, making the resemblance to sarcoma of bone the more real. Starker<sup>52</sup> discussed subperiosteal hematoma in hemophilia and Echtermacht<sup>53</sup> described a 13-year old boy with a huge hematoma associated with the left tibia that was mistaken at first for tumor. The patient died three days after amputation.

### *Hematuria*

Attacks of hematuria are one of the most frequent hemorrhagic episodes in hemophilia, in fact almost 90 per cent of the patients in our series have had one or more episodes. Recurrent attacks are very common. The attacks are usually spontaneous but occasionally follow direct trauma to the kidney region. In one instance an attack was apparently induced by a prolonged train ride, the patient being frequently jarred while sitting up in the coach.

The onset of hematuria is usually symptomless except for the appearance of red urine. Occasionally pain may herald the beginning of the attack or may occur at any time during the course, but it is most common toward the end. The pain is due to the passage of clots, and its location and character depend upon the site of

clot formation. Generally the pain is typical renal colic indicating that the bleeding is from the kidney or pelvis. Following a bout of pain a clot is sometimes passed during micturition and is usually accompanied by severe dysuria.

An attack of hematuria may last a day or so, or may be prolonged for several weeks, no known factor or form of treatment apparently affecting the duration.

Initial or terminal hematuria are seen occasionally as a complication of disease in the anterior or posterior urethra. The spontaneous hematuria of hemophilia may be confused with any one of the other causes of the symptom for which investigation should be made, particularly if the hematuria frequently recurs. Weil<sup>54</sup> believes that hematuria in hemophilia is in most instances caused by the presence of stone. The frequent occurrence of hematuria in the disease makes this appear unlikely.

Although the urine may become quite dark, the actual loss of blood during an attack of hematuria is usually not enough to alter significantly the blood hemoglobin content.

#### *Pharyngeal and Laryngeal Hematomata*

Hematoma formation beneath the mucosa of the pharynx and larynx is one of the few emergencies in hemophilia because of the rapid occurrence in some of the few emergencies in hemophilia because of the rapid occurrence in some of airway obstruction. Fortunately this does not occur very often, although one of the five deaths in this series was from this cause. Baird and Fox<sup>55</sup> found seven instances of this complication reported, in four of whom tracheotomy was not performed and who recovered, while three died following this operation. In their own case tracheotomy was done with recovery.

The patient usually complains first of sore throat, loss of voice, or both. With either of these symptoms careful examination of the larynx and pharynx must be made at once. Sometimes the bleeding begins as an obvious swelling on the posterior pharyngeal wall, but more commonly it cannot be seen except by indirect laryngoscopy. In the latter case the hemorrhage frequently discolors the mucous membrane over the arytenoids and spreads down the laryngeal wall to the false and true cords. Fortunately, obstructive symptoms have not occurred in most of our patients, but when they do they may either appear within a few hours or be delayed for a day or so.

It has been our custom to hospitalize each of these patients at once and to keep a tracheotomy kit readily available (see section on Treatment).

Although most instances of pharyngeal and laryngeal hematoma formation appear to be spontaneous, it may follow overvigorous use of the voice, against which hemophiliacs should be cautioned.

#### *Pulmonary and Pleural Complications*

Pulmonary and pleural bleeding are uncommon complications in hemophilia, although mediastinal and pleural shadows appearing in roentgenograms, presumably from fresh or old hematoma, have been reported.<sup>56</sup> Massive hemothorax and hemoptysis are rare.<sup>57</sup> These complications were not observed in our patients.

*The Acute Abdomen in Hemophilia*

Not only are the usual acute abdominal conditions a problem in hemophilia because of the high operative mortality,<sup>15</sup> but in addition, certain forms of intra abdominal and retroperitoneal hemorrhage so resemble acute surgical emergencies that the greatest diagnostic acumen and surgical caution must be exercised to avoid a fatal result

All the common acute abdominal conditions such as acute appendicitis, acute cholecystitis, perforated peptic ulcer, acute pancreatitis, etc., may, of course, appear in hemophiliacs. Although it is difficult to ascertain the degree, bleeding from or into the damaged tissue may complicate these acute abdominal conditions by increasing the symptoms and delaying healing. Where infection is present it may travel with the bleeding, and in this way spread much farther than it otherwise would. Therapeutic procedures will be discussed in the section on treatment.

Hemophiliacs, in addition, suffer a variety of purely hemorrhagic intra abdominal episodes which both closely mimic and are more frequent than the usual acute abdominal emergencies. In many instances such hemorrhagic episodes are difficult, if not impossible, to differentiate from the common forms of the acute abdomen. Sometimes the course of the illness establishes whether it is purely hemorrhagic or not, but all too frequently the differentiation is obscure and it is extremely difficult to decide not to perform a highly dangerous operation.

Severe upper abdominal pain, usually cramp-like, but sometimes steady and resembling a penetrating or even perforated ulcer, is occasionally seen. The onset is usually progressive over several hours with pain reaching great severity and usually associated with nausea and vomiting. The abdomen may become distended, with upper abdominal tenderness or even generalized tenderness and a board like rigidity. Moderate leukocytosis is usual. The acute condition usually lasts from one to two days and then gradually subsides over a period of several days or occasionally recurs. To place the bleeding accurately in these episodes is usually difficult. In some instances, intraperitoneal bleeding becomes evident by the appearance of free fluid in the peritoneal cavity, together with signs of acute blood loss. A positive benzidine or guaiac reaction in the stool a day or so after the beginning of the episode indicates bleeding into the gastro-intestinal tract, which may be due only to mucous membrane bleeding from persistent retching and vomiting. Massive melena may sometimes complicate this upper abdominal bleeding syndrome, but hematemesis is rare.

Pain in the midabdomen, usually cramplike, and resembling small bowel obstruction is a distressing although uncommon complication in hemophilia and is probably due in most instances to bleeding into the bowel wall, the mesentery, or both, and sometimes associated with intra-abdominal bleeding. Moderate distention and vomiting are the rule and are due to paralytic ileus.

Low abdominal pain is the commonest of the abdominal emergencies in hemophilia. Two apparently unrelated forms of bleeding may occur into the colon wall or the mesocolon, or into or around the ileopsoas muscle. In the first instance, bleeding into the colonic wall or mesentery, the signs are usually those of partial

bowel obstruction vomiting, cramplike abdominal pain, and abdominal distention. A tender, low intra-abdominal hematoma usually forms after a day or so, and finally after several days it may discharge its contents into the bowel with the sudden appearance of melena. Patients exhibiting this condition have been described by Vance<sup>59</sup> and Platou and Platou.<sup>60</sup>

Retroperitoneal bleeding is more common in the low abdominal syndrome than that associated with the colon, and is usually due in these instances to bleeding into or around the ileopsoas muscle. The fact that 15 of our 40 patients had at least one episode of ileopsoas hemorrhage illustrates its frequency and importance as a complication of hemophilia. The syndrome has been described by Birch,<sup>61</sup> Günther,<sup>62</sup> and Fallroth.<sup>63</sup> When on the right side, the ileopsoas hemorrhage resembles acute appendicitis, although the pain seldom begins in the epigastrium. At first the pain is mild but usually in the course of hours becomes severe. Tenderness to palpation and percussion are often exquisite over McBurney's point and rebound tenderness is the rule. There may also be tenderness on rectal examination on the affected side. Leukocytosis is almost always present but usually is moderate. The blood loss is seldom sufficient to produce anemia or signs of acute blood loss. A mass due to a retroperitoneal hematoma often appears within 24 to 48 hours and may be mistaken for an appendiceal abscess, even though the latter seldom appears this early after the onset of the symptoms. Occasionally the hematoma spreads distally down the ileopsoas muscle and may become palpable at Poupart's ligament or even in the femoral canal. When this occurs, differentiation from acute appendicitis becomes easier.

Further aid in differentiating ileopsoas hemorrhage from other intra-abdominal conditions is the distressing complication of partial or complete involvement of the femoral nerve. This usually begins with pain on the anterior surface of the thigh and may be observed soon after the onset of the bleeding. A positive 'psoas sign' may be seen at this time and the hip is usually held in partial flexion. Paresis may be seen at this time and the hip is usually held in partial flexion. Paresis and usually partial or complete anesthesia often follows within two or three days and weakness or paralysis of the thigh extensors with subsequent muscular atrophy follows. As mentioned above, the acute episode lasts as a rule for but a few days, but the mass when present may disappear slowly or even may remain permanently. Likewise, the femoral nerve damage is slow to heal and hypesthesia, muscular weakness and atrophy may be permanent.

#### *Neurologic Complications in Hemophilia*

Spontaneous intracranial hemorrhage is rare in hemophilia<sup>64</sup> in contradistinction to purpura hemorrhagica in which it is the most common cause of death.<sup>9</sup> Bleeding into or around the spinal cord is likewise seldom seen in hemophilia although retroperitoneal hemorrhage sometimes impinges upon a nerve root as it emerges from the spine producing typical unilateral radicular pain.

Peripheral nerve lesions of varying severity and location are very common and usually complicate hemorrhage into a joint or muscle which is in close proximity to the nerve. Thus, the ulnar and superficial peroneal nerves are frequently damaged



in this way Retroperitoneal ileopsoas hemorrhage affecting the femoral nerve is discussed above in the section on the acute abdomen A very complete review of the neurologic complications of hemophilia is to be found in an article by Aggeler and Lucia<sup>65</sup>

### THERAPY IN HEMOPHILIA

In addition to the many manifestations of hemorrhage itself, bleeding in hemophilia may complicate other coexistent diseases The treatment of these primarily nonhemorrhagic conditions may be further complicated by secondary hemorrhage Treatment is directed both to rectifying the diminished blood coagulability locally and systemically, as well as to whatever nonhemorrhagic condition may be present

1 *Blood coagulants* For generations man has been searching for methods to stop bleeding and the number of remedies, both household and medical, attest both the frequency of the problem and the general inefficacy of the methods of hemostasis In an effort to halt the excessive bleeding in hemophilia, a great many remedies have been described, most being for parenteral administration<sup>66-79</sup> We have had little or no experience with most of these therapeutic agents, many of which have been proven ineffective Since Weil<sup>80</sup> in 1905, found that the therapeutic effect of blood transfusions in hemophilia was due to bringing the coagulation time to or near normal, this form of therapy has not only passed the test of time,<sup>81 82</sup> but also is the most physiologic of all the parenteral remedies tried However, even when the coagulation time is brought to normal with blood transfusions the bleeding may continue

Since the antihemophilic activity of both blood and plasma gradually disappears when preserved even at refrigerator temperatures<sup>83</sup> it has been our policy to use human whole blood or plasma not over 24 hours old unless, in the case of plasma, it has been separated soon after phlebotomy and preserved in the frozen state Lyophilized plasma has been shown to be active,<sup>84</sup> but for optimum effectiveness it can not always be depended upon, as several days often elapse between the drawing of the blood and its processing

In the case of acute blood loss of significant proportions either externally or into the tissues, fresh whole blood is the choice, for it not only provides antihemophilic activity but replaces the loss in both red cell and plasma volume Plasma, fresh or frozen, is simpler because cross-matching is not required and it is as rich as whole blood in antihemophilic activity It has been our custom to administer whole blood in the amounts dictated by the severity of the blood loss If whole blood is not necessary, plasma is given in 100 cc to 250 cc quantities for its antihemophilic properties The reduction in coagulation time is usually to or near to normal This effect persists for 6 to 12 hours at the minimum and then the clotting time gradually rises to its preinjection level in the course of another 6 to 12 hours<sup>27</sup> (fig 1) Thus, for continued effect on the coagulation time of the hemophilic patient, blood or its products should be given once or perhaps twice daily during the period of active bleeding

A hemophilic may vary considerably from time to time in his response to antihemophilic material of known potency It is important to determine the coagula-

tion time shortly after the administration of the antihemophilic agent, e g,  $\frac{1}{2}$  hour, and again at a 6- to 8-hour interval, in order to follow the extent and duration of the effect. If the coagulation time does not reach or remain at or near normal the administration should be repeated.

As described above in the section on the coagulation defect in hemophilia, blood plasma fractionation has led to the production of a preparation of human fibrinogen which contains antihemophilic activity and which can be given intravenously to patients with hemophilia.<sup>27</sup> In the dosage recommended there have been no significant reactions observed, and no reported cases of serum jaundice have occurred. \*In addition to absence of icterogenic properties, the material has the ad-

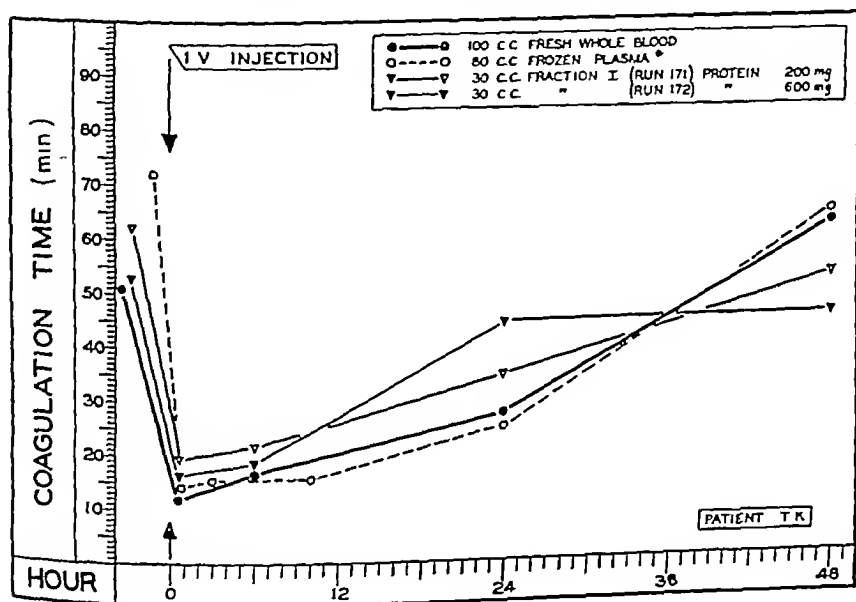


FIG. 1

advantages over whole blood and blood plasma that very small amounts need be administered for maximum effect and that it can be easily and quickly given. There is a great deal of variability in the antihemophilic activity of Fraction I as now available, and there are, in fact, instances when fresh whole blood is more effective in reducing the coagulation time. Thus, the material is in no sense a cure for hemophilia, but its production is a step toward finding a potent therapeutic substance and hopefully a prophylactic material which could be given in hemophilia much as insulin is to a diabetic. For many years such a preparation has been the dream of both hemophiliacs and investigators. In his lectures to students, Dr. Minor has often referred to this goal. At present the limitations in the avail-

\* Since this paper has been submitted for publication two cases of hepatitis probably transmitted with the administration of Fraction I have been observed.

able quantity of Fraction I and problems of stability and route of injection have prevented its use as a prophylactic. Nevertheless, attempts at maintenance of a reduced blood coagulation time have been made by injecting fresh<sup>85</sup> or lyophilized<sup>86</sup> plasma once a week or more often. Significant prolongation of the interval between hemorrhage has been obtained in this way.

The refractory state to blood and its derivatives referred to in the introduction follows, in some instances, the repeated administration of blood, plasma, or the antihemophilic globulin fraction, and arises during or promptly after an hemorrhagic episode, although occasionally it is spontaneous.\* The exact nature of this refractory state is still obscure, but recent work has suggested that there may be a production of antibodies to the antihemophilic substance.<sup>81</sup> This observation has yet to be confirmed.

2. *Rest and exercise* Although strict precautions must be taken by the hemophilic against trauma, this does not mean that he should live a sheltered, inactive life. Heavy manual labor, prolonged fatiguing exercise, the more vigorous sports, and other activities that require severe physical exertion should not be attempted, however, moderate activity should be encouraged depending upon the physical capabilities of the individual, for it not only gives the individual a sense of equality with his associates, but also helps to maintain muscle tone and joint mobility. It is our impression that the decrease in muscle size and tone which occurs with immobilization and disuse<sup>88</sup> may be an important factor in initiating hemorrhage into the muscles and neighboring tissues. Although it is difficult to evaluate because of the possible cyclic frequency of hemorrhages, bed rest with its attendant inactivity appears to us to be an important predisposing factor to hemorrhage. Thus, a hemophilic confined to bed for an acute hemarthrosis, for example, not uncommonly develops hemorrhage in other parts of the body. Convalescent patients are therefore encouraged to take moderate exercise. The aid of an expert physical therapist should be available for directing exercise, both while the patient is in bed and during ambulatory convalescence.

3. *Use of sedatives and analgesics* The fact that internal hemorrhage in hemophilia is regularly accompanied by severe pain which may last for several days or more, and that repeated episodes may be expected throughout the patient's life makes the choice and use of analgesics difficult and of prime importance. The use of morphine is sometimes necessary, but should be avoided if possible. If it is required, the drug should be administered for as short a period as possible because of the danger of dependence and habituation. Meperidine hydrochloride (demerol hydrochloride), also contributing to addiction,<sup>87</sup> has been useful in our hands, but occasions arise in which only morphine is effective. When it is decided to administer these or similar analgesics, maximum effective doses should be used to control the pain.

Aspirin, often fortified with codeine, is often effective for less severe pain, but in the case of codeine too, care against habituation must be taken since moderate pain may be prolonged for weeks, as for example, following an hemarthrosis. Hypnosis with barbiturates may make pain bearable, especially at night.

\* Presently available evidence suggests that this refractory state may occur more frequently following the administration of the antihemophilic globulin fraction than following the administration of blood or blood plasma. The therapeutic use of the antihemophilic globulin fraction cannot be advised therefore until further studies have eliminated this hazard.

4 *Local treatment of external bleeding* Aside from the parenteral administration of blood and its derivatives to reduce the blood coagulation time, many substances have been produced for use locally at the site of bleeding. Some of these preparations are very poor coagulants, and most of those that are effective at all exert their effect as a thromboplastin. That is, they hasten the coagulation of the blood by action with prothrombin and calcium, resulting in the production of thrombin which converts fibrinogen to fibrin. We prefer thrombin as a coagulant because it directly converts the fibrinogen to form a fibrin clot. We have had excellent results with a thrombin prepared from animal blood<sup>88</sup> <sup>89</sup> Thrombin may also be prepared from human blood<sup>89</sup> and has recently been produced on a large scale as a by-product of the preparation of human serum albumin from plasma.<sup>90</sup>

No matter what local coagulant is chosen, adherence should be made to certain general principles. The wound should be cleaned with as little trauma as possible, debris and clots of blood being gently removed. Approximation of the edges may be desired but should not be made with sutures unless absolutely necessary, as each needle hole is another source of bleeding. Thrombin is applied directly to the site of bleeding and is held there by appropriate pressure dressings. It is important to emphasize that the thrombin must be applied directly to the source of bleeding, if not, it will merely form a blood clot in the wound, keeping it open and preventing approximation of the edges, effective hemostasis, and healing. The two principles of treating superficial wounds in hemophilia, then, are first that a known active coagulant be applied to the bleeding surface, and second, that it be maintained there with some form of pressure dressing.

Some of the earliest surgical experiences with hemophiliacs were with the use of cautery, both chemical and thermal. Poland<sup>86</sup> in 1850 described a patient in whom pure nitric acid stopped bleeding from a traumatic lip lesion on two occasions. Ericksen<sup>91</sup> in 1856 tells of a 34-year old male who developed an hematoma extending from the ankle to the popliteal space. Following incision, bleeding areas were touched with cautery with cessation of bleeding. Gangrene developed, however, and following amputation by ligature and cautery, the patient died.

Although cautery may temporarily stop bleeding, its use is not advised since surrounding tissues are usually destroyed or damaged, leading to a secondary area of slough and an enlarged area of bleeding. Thus, a ten year old boy seen by one of us had been treated by cautery with dichromate for a clean tongue cut. The bleeding stopped, only to recur two days later with renewed vigor from a larger wound, this time being stopped only by the application of thrombin on a gauze pack held in place by sponge forceps.

5 *Surgery in Hemophilia* Operative surgery in patients with hemophilia is hazardous and attended by a high mortality.<sup>92</sup> Friedrich<sup>88</sup> estimated a 35 per cent mortality following major operations, and his estimate is probably a conservative one. The operative treatment of specific conditions will be discussed in subsequent sections.

Medical literature concerning hemophilia is replete with reports of various surgical procedures which have been attempted. In most instances some form of local or parenteral coagulation therapy was used, often in addition to blood trans-

\* Hemostatic Globulin (Dried) furnished by Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

fusions Many of these were listed above (Section 1 Blood Coagulants ) but are not discussed because of their large number and variety and the lack of precise observations of their effectiveness Some have been shown to be ineffective

In spite of the high mortality rate, operations, sometimes of considerable magnitude, have been done Among those reported, some of which were successful but many not, are appendectomy,<sup>93</sup> <sup>94</sup> gastro-enterostomy,<sup>96</sup> partial gastrectomy,<sup>9</sup> arthroplasty,<sup>98</sup> <sup>99</sup> eye enucleation,<sup>100</sup> prostatectomy,<sup>101</sup> nephrectomy,<sup>102</sup> mastectomy,<sup>50</sup> and various amputations<sup>103</sup>

If an operation is decided upon, the free use of preoperative and postoperative blood transfusions and, when possible, the local application of thrombin (Section 4) are the only important additions to careful surgical technics Specific surgical problems will be discussed as they occur in the following sections

6 *Treatment of acute hemarthrosis* When an acute hemarthrosis occurs in the lower extremity, bed rest is necessary, otherwise, the patient may be ambulatory, with a sling or other support if the pain permits Ice bags to the part give some symptomatic relief Pain is usually extreme and analgesia is indicated Compression bandages applied before much swelling has occurred have been found useful by some Aspiration of the fluid blood in the joint is not recommended because of the danger of infection and, moreover, in our hands has failed to shorten convalescence significantly Following aspiration of blood the joint pain is usually greatly relieved but returns again in a very few hours Thrombin preparations (sterile, human) may be injected into an acute hemarthrosis, but this therapy has not yet proven to be of value

It is usually not possible to place the affected joint in optimum functional position during the acute phase nor do we consider it necessary since as soon as signs of reabsorption of blood appear, cautious active movement up to the point of pain may be begun and gradually increased, usually until the former range of movement is attained As convalescence progresses and danger from renewed bleeding diminishes, physical therapy is in the form of radiant heat, and whirlpool baths hasten recovery of function Early active movement and physical therapy are the best preventatives of ankylosis

7 *Treatment of Chronic Hemophilic Arthritis* Treatment of arthrosed or otherwise deformed joints is largely orthopedic and must be undertaken with great care so that hemorrhage is not induced either into the affected joint or at points of pressure The use of plaster casts which are gradually wedged to the desired position has often been successful The amount and frequency of the wedging is distinctly less than in nonhemophilic patients, each spreading of the cast being up to the point of first pain Simple Buck's extension is also frequently useful but the same precautions must be observed

Arthroplasty, like other operative procedures in hemophilia, must be seldom undertaken and then only with full knowledge of the mortality as well as the likelihood of a poor result from bleeding into and around the operative site If operation is decided upon, the suggestions listed under Surgical Treatment may be helpful in avoiding complications

However, in spite of these measures, the joints of patients with hemophilia may

become partially or completely ankylosed with deforming muscle contractures and atrophy. When this happens in the legs, symptomatic calluses usually develop on the feet. Softening and removing these calluses provides only temporary relief, but more prolonged help can be obtained with corrective shoes. Patients with hemophilia can use aids to walking without difficulty, such as canes and crutches, and we have one individual in our series who is successfully wearing a prosthetic for a surgically amputated foot.

8 *Treatment of subcutaneous and intramuscular bleeding, and of pseudo tumor.* Bed rest with immobilization of the part is usually automatically resorted to by a patient with a large hematoma of the soft tissues. Ice bags, as in acute hemarthrosis, provide some relief, and analgesia is often required. Firm pressure from an elastic bandage over the entire area and especially over the bleeding point, if known, may reduce the bleeding. It cannot be overemphasized that a large amount of blood may be lost into the soft tissues without producing what would seem to be commensurate swelling. A continual watch of pulse, blood pressure, and hematocrit must be made so that shock does not occur. Blood transfusions not only supply the antihemophilic factor but also replace blood lost.

Great care must be taken to prevent ulceration of the skin over the hematoma as infection and renewed bleeding may become major therapeutic problems.<sup>104, 105</sup>

Hemophilic pseudo tumor, with necrosis and reabsorption of bone, as well as soft tissues, is a potential hazard when fully developed because of its awkwardness and susceptibility to infection. Amputations have been done for this condition. If undertaken, extreme care must be exerted to see that the blood coagulation time is as close to normal as may be obtained and that the surgery induces the least possible trauma. Thrombin should be placed between the stump and its covering.

9 *Treatment of peripheral nerve lesions.* Little further than the treatment outlined in section 7 and 8 can be done to treat the neuritis that not uncommonly develops during the active phase of intramuscular or subcutaneous hemorrhage. Complete regeneration of nerves may be expected in the course of time in many instances while some will be left with residual nerve damage. Physical therapy to maintain muscle tone and prevent contracture and bony ankylosis of joints is indicated. When splints are applied to avoid contracture, they should be bivalved so that physical therapy may be instituted.

10 *Treatment of hematuria and certain urologic complications.* Bleeding from the genito urinary tract is usually renal in origin and is frequently resistant to treatment, continuing in spite of the repeated administration of fresh blood or its derivatives and satisfactory reduction of the blood coagulation time. Absolute bed rest in the supine position may be tried but in our hands has been largely ineffectual. In occasional patients there may be prompt cessation of bleeding following some form of therapy, but generally after a variable period it ceases spontaneously. Except for the occasional development of a mild blood loss anemia there have been no ill effects from continued hematuria. The ureteral passage of blood clots, particularly frequent when bleeding is decreasing, usually causes severe renal colic and may require the administration of morphine or demerol for relief.

As mentioned above, search should be made, if suspected, for stone tuberculosis,

malignancy, or other causes of hematuria, particularly if repeated episodes of bleeding occur. Cystoscopy may be performed in hemophilia if necessary and if carefully done, but ureteral catheterization or retrograde pyelography may induce submucosal ureteral bleeding and probably should not be performed.

Operative intervention in urologic problems in hemophilia is extremely serious. Barney<sup>108</sup> in 1933 described a case in which following a necessary suprapubic cystotomy, failure to control the bleeding resulted in death. Mertz and Meiks<sup>109</sup> reported a patient who died eight days after a nephrectomy for hydronephrosis in spite of repeated transfusions. Hinman,<sup>101</sup> however, successfully removed a prostate in a 66-year old hemophiliac.

*11 Care of the teeth, dental extraction.* Dental prophylaxis is of paramount importance in the care of the hemophiliac. It is to the advantage of the patient that he be seen regularly and often by his dentist and that prophylaxis and necessary repair be performed at an early date. Cavities can be filled without fear of hemorrhage although care should be taken to avoid undue trauma to the gums.

However, frequently, due to the failure of the patient to seek dental care or reluctance of the dentist to perform the indicated procedures, extraction is necessary. In conjunction with the Department of Oral Surgery, Boston City Hospital,\* the method described below has been successfully employed many times in the last five years.

The plan involves reduction in blood coagulation time by parenteral fresh blood or suitable derivatives, and the application of thrombin with pressure to the socket provided by a partial or complete denture.<sup>12 107 108</sup> By combining these two techniques we have been able to perform dental extractions in hemophiliacs with a progressive reduction in the postoperative bleeding so that at present it is minimal.

Before the extraction is performed an impression is taken of the jaw from which the tooth is to be extracted. From this a well-fitting partial or complete denture is made. Its essential features are a labial flange extending from the main body of the denture across the socket from which the tooth is to be removed, and two wire clasps, one on either side of the denture, that serve to secure it firmly in position. Approximately a week prior to the operation, a thin, tightly fitting band of rubber (orthodontia band) is placed about the neck of the tooth to be extracted. During the succeeding several days this band progresses along the tooth root, partially separating it from the adjacent tissues. At times the band will progress rapidly along the root so that it may be necessary to use two or three such bands in order to keep the soft tissues from reapproximating to the tooth after the band has passed.

An hour or so before the actual extraction the patient is given an amount of antihemophilic globulin sufficient to reduce his coagulation time to 15 minutes, or lower if possible. In the event that this material is not available, fresh whole blood, frozen plasma, or its equivalents in antihemophilic activity, may be used. Similar amounts of antihemophilic globulin are routinely administered on the first, second and third postoperative days.

\* The principles and technic employed are largely the result of the enthusiastic work of Dr. Stephen P. Mallett, Oral Surgeon in-Chief, and his staff, particularly Dr. Phillip H. White. We are indebted to them for the details of this presentation which will subsequently be reported in full.

In the majority of our cases, novocaine has been used as an anesthetic although nitrous oxide-oxygen inhalation anesthesia may be safely employed. In extractions of the maxillary teeth it has been the practice of the operator to infiltrate with a fine gage needle the tissues at the free cuff margin of the gingivae rather than using the more conventional type of infiltration. By so doing, the tissues traumatized are localized in one area, over which the mechanical pressure of the denture will be applied. Mandibular block injections are usually necessary for the removal of teeth from the lower jaw, although in this procedure there is danger of causing pharyngeal hematoma.

An attempt should be made to extract the tooth with as little trauma as possible. On occasions, however, small lacerations of the gums have occurred and the socket septa have been removed without increased bleeding.

After the tooth has been removed the socket may be gently sponged and cleaned. Using dried thrombin, an empty novocaine capsule is then firmly packed into the defect and buttressed with a more solid mechanical filler. An oxidized cellulose preparation\* has proven to be very satisfactory for this purpose. No attempt is made to suture the gum margins. The denture is then inserted into position, care being taken to see that the flange fits firmly over the socket.

In the majority of instances, there will be insignificant postoperative bleeding. If such is the case the denture is not removed for approximately a week. At the end of this time it may be taken out for a short trial period. If oozing still continues, a small amount of dried thrombin is applied to the bleeding surface and the denture reinserted. This is repeated at one- or two-day intervals until complete hemostasis has been obtained. If more vigorous bleeding occurs, the denture may be easily removed at any time, the socket cleaned of old clots and repacked, and the denture reinserted.

It is of utmost importance to have a well-fitting denture. It is uncomfortable to the patient if it fits too tightly and local pressure necrosis may occur. On the other hand, if it fits too loosely sufficient mechanical pressure will not be applied in the appropriate area or the movements of the denture may dislodge the clot and hemostasis will not be obtained. By adding flanges as needed to the original denture it may be used for more than one extraction. However, a new denture has to be made from time to time to compensate for the shrinkage of the soft tissues and resorption of the underlying bone. The unpleasant taste that usually occurs after two or three days' wearing of the denture may be partially alleviated with simple mouth washes.

During the period that the denture is being worn, the patients are permitted to be up and about the ward and to engage in their usual activities. They are able to eat and sleep regularly. Conventional partial or complete dentures can be worn by the hemophiliac without difficulty.

12. *Treatment of pharyngeal and laryngeal hematoma.* The potential seriousness of pharyngeal or laryngeal hematoma lies in its occasional propensity rapidly to occlude the airway. For this reason, if suspected, the diagnosis must be confirmed by a competent laryngoscopist and, if confirmed, the patient should be hospitalized.

\* Oxy-cel provided by Parke Davis & Company, Detroit, Michigan.



so that proper supervision is available. A tracheotomy kit is kept near at hand. The diet should be soft or liquid and absolute voice rest enforced. Administration of fresh blood or its derivatives to reduce the blood coagulation time is essential. Generally within 24 hours the swelling begins to recede and convalescence is then rapid and uneventful. If obstruction of the airway becomes imminent, tracheotomy should be done, with the most careful surgical hemostasis and with the liberal use of blood or its derivatives.

13 *Abdominal surgery* In the discussion above concerning The Acute Abdomen in Hemophilia the difficulty was emphasized of differentiating either intra-abdominal or retroperitoneal hemorrhage from the usual acute abdominal conditions. In this regard, Traum<sup>7</sup> reported a patient who was operated upon with a mistaken diagnosis of peritonitis from a ruptured appendix. An hematoma the size of a child's head was found around the right kidney which was evacuated and packed. The patient subsequently died. Scherk<sup>109</sup> has discussed the differential diagnosis of abdominal symptoms in hemophilia and described a 47-year old hemophiliac in whom a diagnosis of acute appendicitis was made. He was treated without operation in spite of the development of a sausage-shaped tumor in the right lower quadrant which disappeared in eight days. A gangrenous appendix, however, was successfully removed by Prima<sup>94</sup> complicated by a fist-sized hematoma in the wound. Cioran<sup>95</sup> likewise reported the removal of a perforated gangrenous appendix with a good result.

It is impossible to be didactic concerning operative intervention on patients with hemophilia in whom an acute abdomen is suspected. Two important facts may be reiterated, however. Intra-abdominal or retroperitoneal bleeding is far more common in hemophiliacs than are the usual abdominal emergencies. Secondly, major surgery has a very high mortality rate in hemophilia. With these facts in mind an unnecessary operation usually may be avoided. A case in point is that of Platou and Platou<sup>60</sup> concerning an eight-year old hemophiliac who was very ill with signs of intestinal obstruction. He improved following the institution of continuous gastric aspiration. Blood transfusions were administered and operation was delayed from day to day and finally avoided. A diagnosis of bleeding into the bowel wall was made. In our experience this set of circumstances has occurred a number of times and operation has not yet been necessary. Likewise, in patients with pain in the right lower quadrant resembling appendicitis, operation has not been done although their number has been large. An ileopsoas hemorrhage was suspected in each. In view of the work of Crile<sup>110</sup> with the use of massive doses of penicillin in peritonitis resulting from appendicitis, the danger of not removing an acutely inflamed appendix may not be as great as it was formerly considered to be. It is probable that occasionally an acute appendix will be missed by this conservative treatment, but again, the operative risk may be as great or greater than that of an unoperated, acutely inflamed appendix.

14 *Social, economic and psychiatric implications* An hereditary disease with an outlook of life-long partial disability inevitably brings with it a multitude of social, economic and psychiatric problems. It is the physician's duty not only to care for

the hemorrhagic episodes, but, in addition, to consider and advise on such matters as vocation, marriage and children

Rightfully, preventive therapy must begin in childhood as soon as the diagnosis of hemophilia is established. The nature of the disease must be clearly explained to the parents so that they will not only endeavor to prevent hemorrhages but will so orient, care for, and instruct the child that he will grow into as useful and productive a citizen as possible, for only in this way will he be well adjusted, and thus happy. More than most children he must be taught independence and self-reliance and must not depend too much upon his parents. This is often difficult for the rest of the family for the hemophiliac is, of course, subject to frequent bouts of pain which automatically make him the center of attention.

Early in life a vocation must be carefully planned. Too often hemophiliacs grow to adult life with little formal schooling because of frequent illness. Vocational training is likewise scanty so that they are capable only of manual labor for which they are quite unfit. A little consideration of the individual and his bent will indicate whether he is to work chiefly with his brain or his hands. In the latter category, art, architecture, mechanical drawing, watch repairing, electrical and radio work offer opportunities. In some communities vocational training of the kind required by hemophiliacs is available.

Ten of the 28 hemophiliacs over 20 years of age in our series are married and have a total of 13 children. To advise against marriage simply adds another probably unnecessary burden to an already troubled life. However, one can ensure that both partners understand fully the nature of the disease and their responsibility both as to its hereditary implications and to the prognosis for future morbidity. The decision is then left to the individuals concerned. It is certainly well for the prospective bride to have a possible gainful vocation in case of prolonged illness of her husband, but many of our hemophiliacs have been able by careful planning to provide an adequate home.

The hemophiliac is continually exposed from an early age to those who feel sorry for him, want to help him, or even consider him an inferior. In addition, he has frequent illnesses and must bear considerable pain. It requires a strong mental constitution to become adjusted to such a life. Fortunately, most hemophiliacs accept their additional burdens as they come and in this way each period of stress builds a better adjusted individual. The physician by frequent discussions is in a position to aid greatly the individual's own effort to learn to live with his disease.

#### REFERENCES

- <sup>1</sup> OTTO J. C. An account of an hemorrhagic disposition existing in certain families. *Med. Repository* 6: 1, 1803.
- <sup>2</sup> RUHRAH J. John Conrad Otto 1774-1844. A note on the history of hemophilia. *Am. J. Dis. Child* 47: 1335, 1934.
- <sup>3</sup> KRUMHAAAR E. B. John Conrad Otto and the recognition of hemophilia. *Bull. J. Hopkins Hosp* 46: 123, 1930.
- <sup>4</sup> MINOT G. R. AND LEE R. I. The blood platelets in hemophilia. *Arch. Int. Med.* 18: 474, 1916.
- <sup>5</sup> BULLOCK W. AND FILDES P. Hemophilia. *Treasure of Human Inheritance*. Francis Galton Eugenics Laboratory Memoirs Univ. of London p. 169. London: Cambridge University Press, 1911.

- <sup>6</sup> BIRCH, C A Hemophilia Clinical and Genetic Aspects Urbana Univ Illinois Press, 1937
- <sup>7</sup> HOWELL, W H Hemophilia Bull N Y Acad Med, 15 3 1939
- <sup>8</sup> STETSON, R P AND LOZNER, E L Hemophilia The Oxford Medicine New York, Oxford Univ Press p 799 1941
- <sup>9</sup> QUICK, A J The Hemorrhagic Diseases and the Physiology of Hemostasis Baltimore, Charles C Thomas, 1942.
- <sup>10</sup> DAVIDSON E C, AND McQUARRIE I Hemophilia A study of the blood clinical course, and the heredity in three cases Bull J Hopkins Hosp 36 343 1925
- <sup>11</sup> ELEY, R C Hemophilia Med Clin North America 21 375 1937
- <sup>12</sup> MILLS, C A Hemophilia J Lab and Clin Med 17 932, 1932
- <sup>13</sup> MACFARLANE, R G The treatment of hemophilic hemorrhage St Bartholomew's Hosp Rep 47 229 1935
- <sup>14</sup> KARK, R Recent developments in hemophilia Clinics 2 15 1943
- <sup>15</sup> POHLE, F J, AND TAYLOR F H L The coagulation defect in hemophilia The effect in hemophilia of intramuscular administration of a globulin substance derived from normal human plasma. J Clin Investig 16 741 1937
- <sup>16</sup> LEE, R I, AND WHITE, P D A clinical study of the coagulation time of blood Am. J Med Sci 145 495, 1913
- <sup>17</sup> JOBLING J W AND PETERSON, W Nature of serum antitrypsin J Exper Med 19 459, 1914.
- <sup>18</sup> LEWIS J H TAGNON H J, DAVIDSON C S, MINOT, G R, AND TAYLOR, F H L The relation of certain globulin fractions of the plasma globulins to the coagulation defect in hemophilia Blood 1 166, 1946
- <sup>19</sup> TOCANTINI L M Relation of contracting surface and anticephalin activity to the maintenance of the fluidity and coagulability of blood Blood 1 156, 1946
- <sup>20</sup> MUNRO, F L, AND JONES, H W Detrimental effect of frequent transfusions Am J Med Sci, 216 710, 1943
- <sup>21</sup> CRADDOCK, C G AND LAWRENCE J S Hemophilia A report of the mechanism of the development and action of an anticoagulant in two cases Blood 2 505 1947
- <sup>22</sup> EPSTEIN R D AND TAYLOR F H L Unpublished data
- <sup>23</sup> PATEK A T JR, AND TAYLOR F H L Hemophilia Some properties of a substance obtained from normal human plasma effective in accelerating coagulation of hemophilic blood J Clin Investig- 16 113 1937
- <sup>24</sup> VAN CREVELD, S Sporadic hemophilia and pseudo-hemophilia Acta Paediatrica 29 37 1941
- <sup>25</sup> LOZNER E L, AND TAYLOR F H L The coagulation defect in hemophilia Studies of the clot promoting activity associated with plasma euglobulin in hemophilia J Clin Investig 18 821, 1939
- <sup>26</sup> TAYLOR F H L, DAVIDSON C S TAGNON H J ADAMS M A MacDONALD A H AND MINOT G R Studies in blood coagulation the coagulation properties of certain globulin fractions of normal human plasma in vitro J Clin Investig 24 698 1945
- <sup>27</sup> MINOT, G R, DAVIDSON C S LEWIS, J H, TAGNON H J, AND TAYLOR F H L The coagulation defect in hemophilia, the effect in hemophilia of the parenteral administration of a fraction of the plasma globulin rich in fibrinogen J Clin Investig, 24 904 1944
- <sup>28</sup> LOZNER, E L, KARK R AND TAYLOR, F H L The coagulation defect in hemophilia The clot promoting activity in hemophilia of berkfelded normal human plasma free from fibrinogen and prothrombin J Clin Investig 18 603, 1939
- <sup>29</sup> LEWIS, J H SOOLIER J P AND TAYLOR F H L Chemical clinical and immunological studies on the products of human plasma fractionation XXXIII The coagulation defect in hemophilia The effect in vivo and in vitro on the coagulation time in hemophilia of a prothrombin and fibrinogen free normal plasma and its derived protein fractions J Clin Investig 25 8761 1946
- <sup>30</sup> LOZNER E L JOLLYFFE L S AND TAYLOR F H L Hemorrhagic diathesis with prolonged coagulation time associated with a circulating anti-coagulant Am J Med Sci 199 318 1940.
- <sup>31</sup> QUICK A J The diagnosis of hemophilia Am J Med Sci 202 469 1941
- <sup>32</sup> BOGGS R Spontaneous hemophilia Report of six cases in brothers Am J Med Sci 181 811 1934-
- <sup>33</sup> HALDANE J B S, AND PHILIP U The daughters and sisters of hemophiliacs J Genetics 31 193

- <sup>21</sup> MACKLIN M T Sex ratios in families with hemophilia *Am J Dis Child* 58 1215, 1937
- <sup>22</sup> STEINER, W R Hemophilia in the Negro *J Hopkins Hosp Bull* 11 44 1900
- <sup>23</sup> CRANDALL N F Hemophilia in the Negro *Am J Med Sci* 192 745-51, 1936
- <sup>24</sup> PACHMAN D J Hemophilia in Negroes Three cases and two geneologic charts *J Pediat* 10 809, 1937
- <sup>25</sup> CAMPBELL, E P Hemophilia in the Negro—report of a case *Med Ann Dist of Columbia* 8 294 1939
- <sup>26</sup> BRUCH H Blut, Blut, und Blutungs Krankheiten *Monatschr f Kindern* 77 273 1939
- <sup>27</sup> KÖNIG F Die Gelenkerkrankungen bei Blutern mit besonderer Berücksichtigung der Diagnose *Klinische Vorträge* 36 233 1892
- <sup>28</sup> CAFFEY, J AND SCHLESINGER E R Certain effects of hemophilia on the growing skeleton Some roentgenographic observations on overgrowth and dysgenesis of the epiphyses associated with chronic hemarthrosis *J Pediat* 16 549 1940
- <sup>29</sup> FONIO A Das Blutergelenk mit besonderer Berücksichtigung der Berner Bluter *Arch f Klin Chir* 191 171 1938
- <sup>30</sup> NEWCOMER N B The joint changes in hemophilia *Radiology* 32 573 1939
- <sup>31</sup> LAMY M L Aspect radiologique des ostéo-arthropathies hémophiliques *Bull et mém Soc Méd de Hôp de Paris* 58 45 1942
- <sup>32</sup> KEEFER, C S AND MYERS W K Hemophilic arthritis *New Eng J Med* 208 1183 1933
- <sup>33</sup> McDONALD, E J AND LOZNER E L Hemophilic arthritis, roentgenographic studies in fifteen adult patients with hemophilia *Am J Roentgenol and Rad Therap* 49 405, 1943
- <sup>34</sup> HILL R L, AND BROOKS B Volkmann ischemic contracture *Ann Surg* 103 444 1936
- <sup>35</sup> SHIH H E Volkmann's contracture with report of case *Nat Med J China* 17 315 1931
- <sup>36</sup> HILL R L Volkmann's ischemic contracture *Tr Hawaii Territ Med App* 26 31 1939
- <sup>37</sup> LORD J P Hemophilia with Volkmann syndrome report of case *J A M A* 87 406 1926
- <sup>38</sup> FIROK W M AND WOODHALL, B Hemophilic pseudotumor diagnosis, pathology and surgical treatment of hemophilic lesions in the smaller bones and joints *Bull Johns Hopkins Hosp* 59 237, 1936
- <sup>39</sup> STARKER L Knochenusur durch ein hemophiles subperiostales Hæmatom *Mitt a d Grenzgeb d Med u Chir* 31 381 1918
- <sup>40</sup> ECHTERNACHT A P Pseudotumor of bone in hemophilia *Radiology* 41 4651 1943
- <sup>41</sup> WEIL P E Les hématuries des hémophile étude pathogénique *Presse Med* 48 468 1940
- <sup>42</sup> BAIRD, K H AND FOX M S Severe sublingual and paratracheal hemorrhage in hemophilia with recovery following tracheotomy *J Pediat* 23 90 1943
- <sup>43</sup> PENDERGRASS E P, AND NEUHAUSER, E B D Pleural lesions in hemophilia Report of a case *Am J Roentgenol* 48 147, 1942
- <sup>44</sup> FREEDMAN P LEVINE S, AND SOLIE-COHEN L Hemothorax in blood dyscrasias *Am J Med Sci* 205 692, 1943
- <sup>45</sup> FRIEDRICH H Über das Operationsrisiko bei Hämophilen *Chirurg* 7 73 1935
- <sup>46</sup> VANCE C A Surgery in hemophilia *Ann Surg* 109 872, 1939
- <sup>47</sup> PLATOU E S, AND PLATOU R V Hemophilia with intestinal obstruction *Minn Med J* 23 857 1940
- <sup>48</sup> BIRCH C LAF Hemophilia *J A M A* 99 1566, 1932
- <sup>49</sup> GUNTHER, H Concerning crippling in hemophilia *Monatschr f Psychiat u Neurol* 91 33 1935
- <sup>50</sup> TALLROTH, A Hemophilia with spontaneous hemorrhage in the iliopsoas muscle followed by injury to the femoral nerve *Acta Chir Scandinav* 82 1 1939
- <sup>51</sup> MARK P F Cerebral hemorrhage in hemophilia *Yale J Biol and Med* 25 185 1942
- <sup>52</sup> AGGELER, P M AND LUCIA S P The neurologic complications of hemophilia *J Nerv and Ment Dis* 99 475 1944
- <sup>53</sup> POLAND A Hemorrhagic diathesis actual cautery recovery *Gux s Hosp Rep London* -5, vii 329 1850-51
- <sup>54</sup> GRANT L On hemophilia and its treatment *Lancet* 2 1-79 1904
- <sup>55</sup> WEIL P E Serum treatment of hemophilia *Lancet* 2 300 1900
- <sup>56</sup> VINES H W C Anaphylaxis in treatment of hemophilia *Quart J Med* 13 -57 1900

- <sup>70</sup> WEIL P Cure of familial hemophilia Abst J A M A 77 737, 1921
- <sup>71</sup> CADDY, L D AND SHRADER, E L The effect of intravenous administration of sodium citrate in two cases of hemophilia J Lab and Clin Med 9 618 1924
- <sup>72</sup> TRAUM E Use of nateina in treatment of large spontaneous hemorrhages into bed of kidney in hemophilia Deutsche Ztschr f Chir 215 351 1929
- <sup>73</sup> MATHE G A AND MITCHELL, V The management of the true bleeder in urological surgery J Urol 21 401 1929
- <sup>74</sup> ELEY P C AND CLIFFORD, S H Hemophilia Treatment by protein sensitization Am J Dis Child 42 1331, 1931
- <sup>75</sup> LAWTON G B JACKSON, W P AND GARDNER J E A case of hemophilia treated by venesection J A M A 95 1443 1932
- <sup>76</sup> ELEY R C Hemophilia The anaphylactic treatment of acute emergencies by passive sensitization New Eng J Med 206 998 1932
- <sup>77</sup> CAMBROOK J D Snake venom and its use in dental hemorrhage Proc Royal Soc Med 29 281, 1936
- <sup>78</sup> ELEY R C GREEN A A AND MCKILHANN, C F The use of a blood coagulant extract from the human placenta in the treatment of hemophilia J Pediatr 8 135 1936
- <sup>79</sup> PAOE R C RUSSELL H K AND ROSENTHAL R L Effect of oxalic acid intravenously on blood coagulation time in three hemophiliacs Ann Int Med 14 78 1940
- <sup>80</sup> WEIL P E L hemophilic pathogenie et serotherapie Presse Med 13 673 1905
- <sup>81</sup> SAHLI H Weitere Beitrage zur Lehre von der Hamophilie Deutsch Arch f Klin Med 99 518, 1910
- <sup>82</sup> ADDIS T The pathogenesis of hereditary hemophilia J Path and Bact 25 427 1911
- <sup>83</sup> TAYLOR F H L LOZNER E L DAVIDSON C S, AND TAONON, H J Preservation of normal human plasma in the liquid state II Comparative *in vitro* studies on the physiologic activity of labile constituents of liquid and frozen plasma J Clin Invest 23 351 1944
- <sup>84</sup> JOHNSON J B The management of hemophilia with lyophilic human plasma intravenously injected J A M A 118 799 1942
- <sup>85</sup> ALEXANDER B AND LANDWEHR G Studies on hemophilia I The control of hemophilia by repeated infusions of normal plasma J A M A (In press)
- <sup>86</sup> DETTRICK J E WHEDON G D AND SHORR E Effects of immobilization upon various metabolic and physiologic functions in normal men Am J Med 4 3 1948
- <sup>87</sup> WIEDER H Addiction to mepheridine hydrochloride (demerol hydrochloride) J A M A 132 1066 1946
- <sup>88</sup> LOZNER E L MACDONALD H FINLAND M AND TAYLOR F H L The use of rabbit thrombin as a local hemostatic Am J Med Sci 202 593 1941
- <sup>89</sup> ADAMS M A AND TAYLOR F H L The thrombin activity of a globulin fraction of the plasma protein of beef swine and human blood Am J Med Sci 205 538, 1943
- <sup>90</sup> EDSALL J T FERRY R M AND ARMSTRONG S H, JR Chemical, clinical, and immunological studies on the products of human plasma fractionation XV The proteins concerned in the blood coagulation mechanism J Clin Invest 23 557 1944
- <sup>91</sup> ERICSEN J E Extravasation of blood into the calf of the leg, hemorrhagic diathesis actual cautery and arrest of bleeding Lancet London 1 511 1856
- <sup>92</sup> KOOREMAN, P J AND HECHT E Chirurgische Eingriffe bei Haemophilen Acta Chirg Scandinav 88 49, 1943
- <sup>93</sup> HIPSLEY P L Recovery from hemophilia after operation for ruptured appendix M J Australia, 1 584 1923
- <sup>94</sup> PRIMA C Gangrenous appendicitis in patient with hemophilia Zentralbl f Chir 88 2152, 1931
- <sup>95</sup> GIORAN S Rare case of successful operation in hemophilia with condition unknown at start Wien Mediz Woch 85 1037 1935
- <sup>96</sup> EMILE WEIL P Les interventions chirurgicales chez les hemophiles Presse Med 39 1021, 1931
- <sup>97</sup> WOJNISENENSKY W Fatality due to hemorrhage in hemophiliac following partial resection of stomach Zentralbl f Chir 5 1872, 1932
- <sup>98</sup> TAVERNIER L Surgery in hemophiliacs value of pre-operative blood transfusion Lyon Chir 11 688 1938
- <sup>99</sup> KORREMAN P J AND HECHT E Surgery in hemophiliacs Acta Chir Scandinav 88 49 1943

- <sup>100</sup> ALTER, F W Post-operative hemorrhage in hemophiliac following enucleation of eyeball *Ophth Rec* 55 512, 1916
- <sup>101</sup> HINMAN, F Prostatectomy in a hemophiliac *S Clin N Am* 13 63, 1933
- <sup>102</sup> MERTZ, H O AND MEIKS L T Hemophiliac as surgical risk Case of nephrectomy with death *Urol and Cutan Rev* 42 557, 1938
- <sup>103</sup> BLALOCK A Amputation of arm of patient with hemophilia *J A M A* 99 1777, 1932
- <sup>104</sup> FIESSINGER N AND MONTAG R Wound of hemophiliac in war *Lyon Chir* 14 738 1917
- <sup>105</sup> POHLE, F J AND MADDOCK S Maggot therapy in an infected wound in hemophilia *J A M A* 109 2055, 1937
- <sup>106</sup> BARNEY, J D The urological aspects of hemophilia Report of a case of hematuria *Boston Med and Surg J* 189 486 1923
- <sup>107</sup> DALITZCH W W Dental extraction in hemophilia *J Am Dent Assoc* 21 1804, 1934
- <sup>108</sup> BIRCH C L , AND SNIDER, F F Tooth extraction in hemophilia *J Am Dent Assoc* 26 1933, 1939
- <sup>109</sup> SCHERK, G Abdominal symptoms in patient with hemophilia *Med Klin* 26 1227, 1930
- <sup>110</sup> CRILE, G , JR Peritonitis of appendiceal origin treated with massive doses of penicillin, Report of 50 cases *Surg Gynec and Obstet* 83 150 1946

# STUDIES ON AN UNDETERMINED CIRCULATING ANTICOAGULANT CASE REPORT AND LABORATORY FINDINGS

By D G DIETER, M D , M SPOONER, M A , F J POHLE, M D †

## INTRODUCTION

IN 1940, Lozner, Joliffe and Taylor<sup>1</sup> reported the case of a 61 year old male Negro with an undetermined circulating anticoagulant. More recently, Lawrence and Johnson,<sup>2</sup> and Munro<sup>3</sup> have reported studies on male patients, previously diagnosed as hemophiliacs, who developed a circulating anticoagulant following numerous blood transfusions. Madison and Quick<sup>4</sup> presented a case and reviewed several other cases of female patients with hemorrhagic diatheses characterized by prolonged coagulation times.

The circulating anticoagulants present in the patients of Lozner et al,<sup>1</sup> Lawrence and Johnson,<sup>2</sup> and Munro,<sup>3</sup> although never identified, had the following common characteristics: (1) they prolonged the coagulation time of normal blood, (2) they were thermostable, (3) they showed no antithrombic activity, (4) they were not neutralized by protamine, (5) they did not pass through semipermeable membranes, (6) they were not extracted by ether. Lozner and his associates found that the anticoagulant material which they described was not associated with the euglobulin fraction which contained the antihemophilic property of plasma. Munro found that the anticoagulant with which he was working was not precipitated as euglobulin. Munro noted that the anticoagulant, when precipitated as a globulin, maintained an anticoagulant activity equal to that of the plasma from which it was derived. It was also stable in a pH of 6.5 to 11.0. So far as history and clinical observation is concerned, it is relatively certain that the patient described by Lozner and Taylor was not a case of hemophilia.

The patients reported by Madison and Quick<sup>4</sup> and referred to as hemophilia like were probably similar to those already described, although the data presented are insufficient to make a positive statement. The fact that the patients discussed by these authors, in spite of the fact that they may have had different diseases, had an increased coagulation time as the only abnormal finding suggests an anticoagulant with the same characteristics as those investigated by Lozner et al,<sup>1</sup> Lawrence and Johnson,<sup>2</sup> and Munro.<sup>3</sup>

This paper presents the history, hematologic studies, and clinical course of a patient with a prolonged coagulation time due to an undetermined anticoagulant. This anticoagulant appears to be similar to those which were present in the above mentioned cases.

## REPORT OF CASE

The patient, a 68 year old white male banker, was admitted to Madison General Hospital on October 12, 1946 complaining of loss of appetite for five days prior to admission. This was followed by generalized muscular aches, pains in the extreme lower abdomen, and gross hematuria.

From the Departments of Medicine and Physiology, University of Wisconsin Medical School, Madison, Wisconsin.

This study was aided by a grant from the Wisconsin Alumni Research Foundation.

† Deceased.

*History of present illness* The patient had apparently been in good health until eight months before admission at which time he developed what appeared to be a contact dermatitis on the back of his right hand which gradually spread over both arms and back. This was treated with sulfathiazol ointment with no improvement. Two weeks after the onset he was admitted to the State of Wisconsin General Hospital where he was treated with boric acid compresses and boric ointment. He recovered and was discharged on the sixth hospital day.

The patient was readmitted to the Wisconsin General Hospital one month later because of recurrence of the skin lesions. The condition was diagnosed at this time as pemphigus and the patient was treated with stovarsal 50 mg before breakfast for three days. After a three day interval in which no drug was given the dose of stovarsal was increased to 100 mg daily. With this regimen the process slowly subsided and the dose was gradually increased to 250 mg of stovarsal daily for three successive days followed by three day rest periods. The patient was discharged on the sixty-seventh hospital day and followed as an out patient for the next three months. While an out patient, he received the same therapy for two months with complete disappearance of all skin lesions except for a small bullous lesion on the left hand.

Approximately two months prior to admission to Madison General Hospital, the patient developed an acute painful swelling of the left elbow. This was thought to be an acute bursitis. The joint was aspirated and blood was obtained. A pressure bandage was applied and no further bleeding occurred. Later, 2 large ecchymotic areas developed on his right arm while he was bair casting. These gradually subsided.

*Past Medical History* The patient had a benign bladder tumor fulgurated twelve years prior to admission.

*Family History* No other members of the patient's family had had any hemorrhagic diatheses.

*Physical Examination* The patient was a well developed, fairly well nourished man who did not appear acutely ill. The temperature was 99.6 F, the pulse rate was 80, the blood pressure 150/80 mm Hg.

The vessels of the optic fundi showed a grade 2 sclerosis with no evidence of hemorrhage or exudates. A few palpable cervical and axillary nodes were present. There were a few scattered, fine crepitant rales in the posterior lung fields bilaterally. The left cardiac border was percussed 1 cm outside the mid clavicular line. There were grade 1 apical and aortic systolic murmurs which were not transmitted. The liver edge was palpable 2 cm below the right costal margin and was nontender. The spleen was not palpable. There was moderate tenderness over the left side of the abdomen at the level of the umbilicus. Bilateral indirect inguinal hernias were present. Vibratory sense was absent in the left leg. Sternal tenderness was noted.

The preliminary laboratory studies were as shown in table 1.

On cystoscopic examination, blood was seen coming from the left ureter.

X ray studies of the chest were negative for signs of tuberculosis and pneumonitis. A retrograde pyelogram of the left kidney revealed that the kidney was displaced upward. X ray studies of the kidneys, ureter, and bladder taken after the retrograde pyelogram showed small areas of opaque material in the region of the left kidney pelvis which was interpreted as the contrast material of the retrograde pyelogram incorporated in blood clots.

*Clinical course* The patient's stay in the hospital was characterized by exacerbations of bleeding followed by a quiescent period during which the patient improved. On one occasion his condition appeared terminal. Auricular fibrillation developed with evidence of decompensation. The blood nonprotein nitrogen became elevated and symptoms and signs of uremia followed. There was a gradual recovery from this acute phase. Numerous episodes of spontaneous hemorrhages occurred which involved the upper and lower extremities. At one time the hemorrhage into the left arm was so extensive that a left radial palsy resulted. There were intracapsular hemorrhages into the shoulder, elbow, hip and knee joints. On two occasions bleeding occurred into the tongue with extension into the sublingual region and the pharynx. There were two episodes of gross hematuria. On several occasions the physical findings were compatible with intra abdominal and retroperitoneal bleeding. There was one episode of severe low back pain associated with clonic contractions of the muscles of the back and both lower extremities. It was thought that this resulted from an extensive hematoma compressing the spinal cord. At one time there was hemorrhage into both parotid capsules. Progressively, however, it appeared that the patient was slowly improving, for the episodes of bleeding were not as frequent and the hemorrhages



were less extensive. Because of the futility of the treatment and the low morale of the patient he was discharged on August 16, 1947. His condition has improved subjectively since discharge. There has been increased appetite. The radial nerve palsy has disappeared. There have been no new episodes of bleeding up to the present time, even though a coagulation time done after two months at home was 90 minutes.

**Therapy.** Along with the supportive measures that were necessary to control pain, combat the persistent anemia and maintain the patient in the best possible state of nutrition and hydration, specific therapeutic agents were employed in an attempt to control the bleeding tendency. Blood transfusions, plasma, concentrated albumin, intravenous calcium gluconate, hemostatic serum, vitamins C, B, P and glucoside of quercetin (rutin) were administered until the condition could be further investigated. When it was discovered by adding the patient's plasma to normal whole blood that the coagulation defect was due to a circulating anticoagulant which prolonged coagulation time of normal blood, 50 cc. of 1 per cent solution of salmine protamine were given intravenously daily for fourteen days.<sup>7, 8</sup> This therapy failed to affect the coagulation time materially.

TABLE 1—*Resume of Laboratory Findings*

Erythrocyte count	1,570,000 to 4,130,000 cells per cu. mm. blood
Hemoglobin	5 to 12 Gm. per 100 cc. blood
Leukocyte count	8,200 to 19,500 cells per cu. mm. blood
Differential	neutrophils increased to 87% during febrile state
Sedimentation rate (Wintrobe)	4.5 mm. in one hour
Blood non protein nitrogen	29-75 mg. per 100 cc.
Hanger's test (cephalin-cholesterol flocculation)	2+ in 48 hours
Fasting blood sugar	87 to 129 mg. per 100 cc.
Icterus index	8 to 50 units
Total serum protein	4.5 to 6.4 Gm. per 100 cc.
albumin	1.5 to 3.1 Gm. per 100 cc.
globulin	1.8 to 4.4 Gm. per 100 cc.
Serology	negative

### METHODS

The experimental techniques used in these investigations were kept as uniform as possible. All blood samples were drawn in cooled, oiled syringes. When citrated plasma was used, the blood was obtained by venipuncture and mixed with 3.8 per cent sodium citrate in the ratio of 9:1. The mixture was kept in an ice bath until used. With the exception of the studies on the effect of high and low centrifuging and platelet activity, the blood was centrifuged at 1500 rpm for ten minutes at 4°C. When uncitrated plasma was used, clustroid tubes were substituted for glass.

The Lee-White method was used in determining the coagulation times of whole blood. In studying the anticoagulant, glass tubes 13 mm. in diameter were placed in a water bath at 37°C. and all clotting times were determined at that temperature. The reagents used in all experiments were also kept at 37°C. The volumes utilized in the individual studies were maintained at 1 ml. except in several specified instances. With a calcium chloride concentration of 0.025 M the coagulation time of recalcified normal plasma was 2-3 minutes, that of normal uncitrated plasma, 4.5 to 5.5 minutes.

*Effect of citrated normal human plasma on the coagulation time of the patient's blood* To determine whether human plasma in minute quantities would shorten the coagulation time of the patient's blood, the proportions were set up as shown in table 3. Small amounts of normal plasma shortened the coagulation time of the patient's blood to some extent, but did not return the coagulation time to normal limits. The coagulation time changed very little when larger amounts of normal plasma were added to the patient's blood.

*Effect of patient's citrated plasma on the coagulation time of normal blood* Amounts of patient's plasma varying from 0.1 ml. to 0.003 ml. were added to 2 ml. of normal blood. In all cases, isotonic salt solution was added in sufficient quantity to make the volume of the patient's blood preparation equal to 0.1 ml. The results are given

TABLE 2.—*Special Hematologic Studies*

Platelet count (direct wet method)	216 000 to 324 000 per cu. mm. blood
Bleeding time (Duke)	1.5 to 3.5 minutes
Coagulation time (capillary tube)	4.5 to 4.5 minutes
(Lee White)	90 to 270 minutes
Clot retraction	normal in 24 hours at 37 C.
Tourniquet test (Rimpel Leede)	normal
Prothrombin concentration (Quick)	91 to 105%
Fibrinogen	normal
Ascorbic acid (fasting whole blood)	0.40 mg. per 100 cc.
(fasting plasma)	0.13 mg. per 100 cc.
Anthr thrombin activity of serum (Wilson)	normal

TABLE 3.—*Effect of Citrated Normal Human Plasma on the Coagulation Time of the Patient's Blood*

	Coagulation Time
	minutes
2.0 ml. patient's blood (control)	127
2.0 ml. patient's blood + 0.01 ml. normal plasma	84
2.0 ml. patient's blood + 0.03 ml. normal plasma	83
2.0 ml. patient's blood + 0.05 ml. normal plasma	68
2.0 ml. patient's blood + 0.10 ml. normal plasma	129
2.0 ml. patient's blood + 0.20 ml. normal plasma	120

in table 4. These data show that an anticoagulant activity was present in the patient's plasma.

*Effect of the patient's uncitrated plasma on normal uncitrated plasma* It was found that when dilutions ranging from 0.05 to 0.20 ml. of the patient's uncitrated plasma were added to 0.4 ml. of normal uncitrated plasma, with 0.15 M sodium chloride added to make a volume of 1.0 ml., the effect, although not as marked as that shown in Tables 4 and 5, showed some tendency toward prolongation.

*Effect of patient's citrated plasma on normal citrated plasma* The above experiment was repeated with citrated normal and patient's plasma to which was added 0.4 ml. of 0.025 M calcium chloride and 0.15 M sodium chloride to make a total of 1.0 ml. The results of this and a control are shown in table 6.

The data show that when increased amounts of the patient's plasma, previously recalcified, are added to 0.4 ml of normal plasma, the coagulation time is increased. The effect is not extremely marked until 0.4 cc of the patient's plasma is added, in which case it is shown that equal amounts of normal plasma and patient's

TABLE 4—Effect of Patient's Citrated Plasma on the Coagulation Time of Normal Blood

	Coagulation Time
	minutes
2.0 ml normal blood (control)	12
2.0 ml normal blood + 0.003 ml patient's plasma	18
2.0 ml normal blood + 0.005 ml patient's plasma	24
2.0 ml normal blood + 0.010 ml patient's plasma	25
2.0 ml normal blood + 0.030 ml patient's plasma	48
2.0 ml normal blood + 0.050 ml patient's plasma	58
2.0 ml normal blood + 0.100 ml patient's plasma	82

TABLE 5—Effect of Patient's Uncitrated Plasma on Normal Uncitrated Plasma

Patient's Plasma	Normal Plasma	0.15 M NaCl	Coagulation Time
ml	ml	ml	sec.
0.00	0.40	0.60	45
0.05	0.40	0.55	70
0.15	0.40	0.45	130
0.20	0.40	0.40	70
0.20	0.00	0.80	1490
0.00	0.20	0.80	45

TABLE 6.—Effect of Patient's Citrated Plasma on Normal Citrated Plasma. *ML of material added to 0.4 ml of normal citrated plasma and recalcified with 0.4 ml of 0.025 M calcium chloride*

Normal Plasma	Patient's Plasma	Control Plasma	0.15 M NaCl	Coagulation Time
ml	ml	ml	ml	sec
0.40	0.00	0.00	0.40	25
0.40	0.05	0.00	0.35	55
0.40	0.15	0.00	0.25	65
0.40	0.20	0.00	0.20	80
0.40	0.40	0.00	0.00	230
0.40	0.00	0.00	0.40	25
0.40	0.00	0.05	0.35	30
0.40	0.00	0.15	0.25	25
0.40	0.00	0.20	0.20	25
0.40	0.00	0.40	0.00	30

plasma give a coagulation time of over 23 minutes. A control experiment, adding recalcified normal plasma to normal plasma did not show this type of change.

Effect of high and low centrifuging on patient's citrated and uncitrated plasma as compared with the normal. To rule out hemophilia further, samples of citrated and un-

citrated patient's and normal blood were submitted to high (3000 rpm for five minutes) and low (1000 rpm for five minutes) centrifuging. Quick<sup>4, 5</sup> has observed that after high centrifuging the coagulation time of recalcified hemophilic plasma is considerably slower than that obtained by spontaneous sedimentation or low centrifugation. This could not be demonstrated on the plasma of this patient. The findings are recorded in table 7.

Samples of citrated and uncitrated blood from the patient and from a normal individual were submitted to centrifugation at 3000 rpm per minute for five minutes and also at 1000 rpm for five minutes. This experiment was done in order to determine whether or not the same relation to spinning blood at 3000 rpm and 1000 rpm, which Quick found in hemophilia, applied to the blood of this patient. No such similarity was obtained.

*Effect of salmine protamine and toluidine blue on the coagulation times of patient's and normal blood*<sup>6-8</sup>. Although it has been stated above that salmine protamine was

TABLE 7—Effect of High and Low Centrifuging on Patient's Citrated and Uncitrated Plasma as Compared with the Normal

	Normal Plasma	Patient's Plasma	0.15 M NaCl	0.025 M CaCl <sub>2</sub>	Coagulation Time
	ml	ml	ml	ml	min
High speed centrifuging of uncitrated plasma	0.00 0.20	0.20 0.00	0.80 0.80	0.00 0.00	68.0 4.0
Low speed centrifuging of uncitrated plasma	0.00 0.20	0.20 0.00	0.80 0.80	0.00 0.00	149.0 4.5
High speed centrifuging of recalcified plasma	0.00 0.20	0.20 0.00	0.60 0.60	0.40 0.40	9.5 2.0
Low speed centrifuging of recalcified plasma	0.00 0.20	0.20 0.00	0.60 0.60	0.40 0.40	11.0 3.0

It was found that by employing the technique outlined in the explanation of methods, normal blood remained unclotted for over 90 minutes and the patient's blood was not coagulated 6 hours later.

used intravenously with no appreciable reduction of the clotting time, experiments were performed to test the effectiveness of it, and toluidine blue in vitro. Amounts of a 0.1 per cent solution of the two drugs, ranging from 0.02 to 0.10 ml, were added to 0.20 ml of the patient's citrated plasma which was diluted to 1.0 ml with 0.15 M sodium chloride and recalcified with 0.40 ml of 0.025 M calcium chloride. It was found that these drugs further prolonged the coagulation time of recalcified patient's plasma. This experiment was repeated using normal blood with similar results.

To test the effectiveness of these drugs to neutralize the anticoagulant properties of heparin, 1 unit of heparin was added to 0.20 ml of normal plasma. The reagents salmine protamine, toluidine blue, 0.15 M sodium chloride and 0.025 M calcium chloride were added in the same order and amounts as discussed in the previous paragraph. These studies showed that 1 unit of heparin prolonged the coagulation time of normal recalcified plasma to 11 minutes (normal 2-3 minutes) and that

0.02 ml of either of the two drugs being tested reduced the clotting time to 4.5 minutes. Amounts in excess of 0.02 ml of salmine protamine and toluidine blue prolonged the coagulation times.

### *Studies on Platelet Fragility*

Studies on platelet fragility were performed according to the method of Muhre, Bogart and Hogan<sup>9</sup> by combining the patient's recalcified plasma with concentrations of sodium chloride varying from 0.33 to 2.5 per cent. The results of these experiments show that the clotting time obtained with 0.8 per cent saline solution was found to be the same as that obtained with recalcified plasma, i.e., 10 minutes.

TABLE 8—*Studies on Platelet Activity*  
Recalcification was carried out in each instance with 0.40 ml of 0.025 M  $\text{CaCl}_2$

Platelet Poor Plasma	Saline Suspension of Platelets	0.15 M NaCl	Coagulation Time
ml	ml	ml	min
0.20 normal	0.20 normal	0.20	2.0
0.20 normal	0.20 patient	0.20	3.0
0.20 normal	0.00 —	0.40	5.5
0.20 patient	0.00 —	0.40	16.0
0.20 patient	0.20 patient	0.20	12.0
0.20 patient	0.20 normal	0.20	10.0

TABLE 9  
Test System: 0.20 ml patient's citrated plasma + 0.40 ml normal plasma + 0.40 ml 0.15 M NaCl + 0.40 ml 0.025 M  $\text{CaCl}_2$

Conditions to which Patient's Plasma was Subjected	Coagulation Time
	minutes
4 degrees C. for 24 hours	3.0
Room temperature for 4 hours	3.0
61 degrees C. for 10 minutes	7.5
Unheated plasma	6.5

When the sodium chloride solution became hypertonic the coagulation time was prolonged. This procedure was repeated on normal plasma with similar findings.

### *Studies on Platelet Activity*

To rule out further the possibility that the platelets were responsible for the coagulation defect, a study of platelet activity was carried out according to the method described by Patek and Stetson.<sup>11</sup> The technique for drawing and citrating the blood was that of previous experiments except that paraffin-coated tubes were used instead of glass. The pipets used in these studies were coated in the inside with a thin film of collodion. Plasma was obtained by centrifuging the blood at 1500 rpm for ten minutes. This plasma was withdrawn and centrifuged at 4200 rpm for fifteen minutes to produce platelet-poor plasma. The platelets were separated

from the platelet-poor plasma and were washed in normal saline and resuspended in a volume of 0.15 M sodium chloride equal to the original volume of plasma. The results of this experiment are shown in table 8.

The data indicate that there was no essential difference in the activity of platelet suspension obtained by this technic and that obtained from normal blood and from the blood of the patient.

*Effect of cold storage, room temperature, and heat on coagulation time of patient's citrated plasma* Citrated samples of the patient's plasma were subjected to 4 degrees Centigrade for twenty-four hours, room temperature for three to four hours, and 61 degrees Centigrade for ten minutes. It was found that heat did not destroy the anticoagulant, but when the plasma was allowed to stand at room temperature for four hours or in a refrigerator at 4 degrees Centigrade for twenty-four hours, the coagulation time of patient's plasma was normal (2-3 minutes) and the anticoagulant action on normal plasma had disappeared as shown in table 9.

#### *Effect of Dialysis*

Ten ml. of the patient's plasma, prepared in the usual manner, were placed in a viscose casing bag and allowed to rotate in distilled water for a period of twenty-four hours at 4 degrees centigrade. The dialysate and the contents of the bag were found to have no anticoagulant activity.

#### *Electrophoretic Analysis*

The  $\alpha/\gamma$  ratio was found to be 0.63. The patient's plasma was submitted to electrophoretic analysis. Increased amounts of each of the globulin fractions were reported. However, as a whole, the pattern of the globulin proteins was not remarkable. The actual analyses for albumin and globulin, based on the Sherring diagram was 2.9 Gm. per cent albumin, 4.6 Gm. per cent globulin per 100 ml. of plasma.

### DISCUSSION

The known hemorrhagic diatheses, such as increased capillary fragility, thrombocytopenic purpura hemorrhagica, afibrinogenemia, hemophilia, pseudohemophilia, hypoprothrombinemia, athromboplastinopenia and afibrinogenopenia have been ruled out either by the history or laboratory findings, as summarized in table 1, or by the therapy that the patient received.

The discovery that the patient's plasma prolonged the coagulation time of normal blood established the presence of a circulating anticoagulant. The investigative results of the patient's coagulation defect do not place this anticoagulant in four of the five categories postulated by Quick,<sup>8</sup> namely, decalcifying agents, antiprothrombins, antithrombins and fibrinogen antagonists. The question of whether antithromboplastin is present is not clearly answered.

The presence of any one of these factors as the cause for the defect in the coagulation mechanism, although not positively ruled out, has at least been eliminated except for antithromboplastin. The studies show that the patient's anticoagulant was not destroyed at 61 C. for ten minutes, while the antithromboplastin described

by Tocantins was destroyed by heat Munro's<sup>3</sup> argument that there possibly exists more than one antithromboplastin is logical

The investigative work conducted on this patient was as complete as possible with the available techniques The data accumulated are similar to those obtained by Lozner et al.,<sup>1</sup> Lawrence and Johnson,<sup>2</sup> and Munro<sup>3</sup> regarding the nature of the anticoagulant with one probable exception, namely, their anticoagulant was stable to storage as well as heat, a finding which is probably not altogether true for this patient, so far as one may judge by a comparison of table 3 and 8 However, if one can compare the data of whole blood and plasma as is necessary in these experiments, one would judge that at least part of the antithromboplastic or anticoagulant activity was destroyed Neither the anticoagulant described by these workers nor the one described here passed through semipermeable membranes It is thought, however, that the results obtained on these studies are comparable to those reported by other investigators on patients with similar afflictions and hence this case probably belongs in the same or a similar category

The factor which precipitated this hemorrhagic diathesis is as obscure as in the case reported by Lozner et al.<sup>1</sup> It is possible that the pemphigus was the exciting factor Stovarsol cannot be eliminated, even though routine laboratory studies during the period that the drug was used were in no way unusual Coagulation times (capillary tube method) of 5 patients with pemphigus, who received stovarsol therapy, were within normal limits Another possibility is that the prolonged coagulation time would have occurred spontaneously Unlike the patients of Lawrence and Johnson<sup>2</sup> and Munro,<sup>3</sup> our patient had a coagulation defect prior to the administration of multiple transfusions

### CONCLUSIONS

The results of various studies upon a patient with a coagulation defect are reported The cause of this defect was found to be a circulating anticoagulant, whose exact nature remains obscure

### ACKNOWLEDGMENT

The authors are indebted to Dr. Ovid O. Meyer for criticisms and suggestions in preparation of the manuscript to Dr. John Malec of Madison General Hospital for permission to use the clinical material to Eli Lilly & Company for the protamine to Dr. P. P. Cohen and Miss Frances Thompson of the Department of Physiological Chemistry for the electrophoretic and fibrinogen analyses

### REFERENCES

- <sup>1</sup> LOZNER, E. L., JOLIFFE, L. S. AND TAYLOR, F. H. L. Hemorrhagic diathesis with prolonged coagulation time associated with a circulating anticoagulant *Am J M. Sc.* 199: 305, 1940.
- <sup>2</sup> LAWRENCE, J. S., AND JOHNSON, J. B. The presence of a circulating anticoagulant in a male member of a hemophilic family *Tr. Am. Clin. & Climatol. A.* 57: 223, 1942.
- <sup>3</sup> MUNRO, F. L. Properties of an anticoagulant found in the blood of a hemophilic. *J. Clin. Investigation* 35: 422, 1946.
- <sup>4</sup> MADISON, F. W., AND QUICK, A. J. Hemophilia like disease in the female *Am J M. Sc.* 119: 441, 1945.
- <sup>5</sup> QUICK, A. J. *The Hemorrhagic Diseases* Springfield Ill. Charles C. Thomas, 1942.
- <sup>6</sup> ALLEN, J. G., AND JACOBSEN, L. O. Hyperheparinemia Cause of the hemorrhagic syndrome associated with total body exposure to ionizing radiation *Science* 105: 388, 1947.

- <sup>7</sup> CHARGAFF E , AND OLSON, A. B. Studies on the chemistry of blood coagulation vi Studies on the action of heparin and other anticoagulants The influence of protamine on the anticoagulant effect in vivo J Biol Chem 122 153 1937
- <sup>8</sup> JACQUES L. B. , AND WATERS E. T. The identity and origin of the anticoagulant of anaphylactic shock in the dog J Physiol 99 454 1941
- <sup>9</sup> MUHRER, M. E. BOGART R. AND HOGAN A. G. Estimation of platelet fragility Am J Physiol 141 449 1944
- <sup>10</sup> LOZNER, E. L. AND TAYLOR F. H. L. The effect of foreign surfaces on blood coagulation J Clin Investigation 21 241 1942
- <sup>11</sup> PATEK A. J. JR. AND STETSON R. P. Hemophilia I The abnormal coagulation of the blood and its relation to the blood platelets J Clin Investigation 35 531, 1958



# FAMILIAL PANMYELOPHTHISIS

## FANCONI SYNDROME IN ADULTS

By KARL ROHR, M D

THE FOLLOWING case histories are of interest because they are the first reports of a familial panmyelopathy of the hypoplastic type occurring in adults. Two brothers were affected.

### CASE REPORTS

*Case 1.* Sch., Franz, was born in 1921. As a boy he was nicknamed "the negro" because of marked pigmentation. He was healthy apart from bouts of eczema.

In 1939, at the age of 18, he had poliomyelitis with resulting weakness of the abdominal muscles. Anemia was discovered for the first time during this illness, with hemoglobin levels varying between 60 and 80 per cent. In April 1945 the hemoglobin was again 60 per cent. In August 1945 the hemoglobin had declined to 53 per cent and in September to 51 per cent. He was given transfusions and Ferredoxin therapy and his hemoglobin rose to 80 per cent. When seen early in 1946 after accidental burning of one arm, his hemoglobin had again dropped to 48 per cent and he was transfused. In June of that year his hemoglobin was 65 per cent. He complained of being very tired and developed dyspnea with little exertion. He also complained of severe pains in the tibiae and vertebrae, slight edema and gingivitis. In November 1946 three teeth were extracted because of stomatitis and this was followed by fever ranging between 38 and 40 C. there was profuse bleeding, the hemoglobin declining to 30 per cent and later to 24 per cent. Temporary improvement followed transfusions and penicillin therapy. Later that year he had bronchopneumonia and his hemoglobin was found to be only 10 per cent. He died in March 1947 at the age of 26 years.

Physical examination showed that the form and the size of the head were normal, as were the genitalia. The skin showed a marked greyish pigmentation, especially on the face, forearms and to a lesser extent on the abdomen. Petechiae were seen in the skin and mucous membranes in 1944 and in November 1946 at the time of the teeth extraction he showed marked pallor, gingivitis, stomatitis and glossitis and there were hemorrhages in the fundi of the eyes. The heart was found to be slightly enlarged and in September 1945 the blood pressure was 130/70. The electrocardiogram was normal at that time but in November 1946 showed signs of myocardial damage.

The urine showed urobilinogen and indican but no porphyrin. Serum bilirubin was 0.3 mg per cent and phosphates and phosphatase were normal. The Takata Ara reaction was negative, the Weleman coagulation time was 0.25 (enlarged) and the serum proteins were 6.8 Gm per cent.

*Hematologic findings.* The course of the anemia has already been indicated and is shown in figure 1. The red cell counts initially were between 2.7 and 3.5 million per cu mm, later dropping to 1.1 to 1.2 million per cu mm and finally to 660,000 per cu mm. The color index varied from 0.96 to 1.39 but was usually over 1.2.

The white blood cells in 1939 were 2,700 to 8,300 per cu mm. During 1945 the count was about 4,000 per cu mm at first, later dropping to between 2,000 and 4,000 per cu mm. In November 1946 the count was only 500 per cu mm and finally reached as low as 310 per cu mm. The polymorphs were 63 per cent at the beginning, with 25 per cent lymphocytes. This gradually changed so that the polymorphs dropped to 58 per cent and then to 34 per cent, the lymphocytes rising to 31 per cent and later to 60 per cent. Monocytes varied between 4 and 10 per cent and the eosinophils between 1 and 3 per cent. The blood smear showed anisocytosis of marked degree throughout the illness, with macrocytosis and microcytosis and poikilocytosis. Polychromasia was marked for a long time. Reticulocytes were 2.4 per cent in June 1946 but later declined to 0.3 and 0.4 per cent.

The platelets were noted to be diminished in August 1945 and counts during the next year lay between 4,600 and 32,000, with a drop finally to 1,000 per cu mm.

From the Medical Department, University of Zurich, Switzerland.

The bleeding time was 5 minutes, and 1 1/2 minutes and the coagulation time was normal on two occasions (5 minutes). The osmotic fragility test showed initial hemolysis in 0.44 per cent NaCl and complete hemolysis in 0.32 per cent NaCl.

The sedimentation rate was first found to be high in 1944. Readings by the Westergren method showed results of 60 to 101 in the first hour and 76 to 130 in the second hour except for readings of 30 for the first hour and 60 in the second hour after transfusions had raised the hemoglobin to 30 per cent. In the terminal stages of the illness the readings were 172 and 175 in one and two hours respectively.

Bone marrow studies during life are of interest. In 1945 smears showed abundant marrow macroblastosis and increase of the metamyelocytes and stab forms. In 1946 the marrow showed a good deal of fat hypocellularity with few basophilic erythroblasts and myelocytes and almost no neutrophils, but a great increase in the reticular cells of the lymphoid and plasma cell types (fig. 2). In 1947 the marrow was even poorer in the normal cell types. Lymphoid and plasma cells predominated, being seen in groups of 6 or 8. Lymphocytes were also increased, in parts a great many fibrocytes with fibril formation were seen, and there were also an unusual number of tissue mast cells as many as 4 or 5 per field (fig. 3).

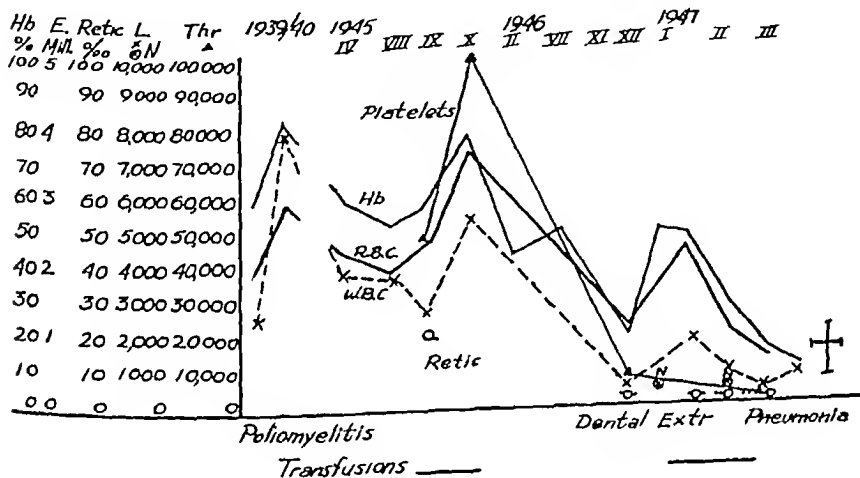


FIG. 1. COURSE OF THE BLOOD COUNTS IN SCH. FRANZ

**Autopsy report.** The heart showed dilatation with hypertrophy of the left side and hemorrhages into the endo- and myocardium. There was bronchopneumonia at the right lower lobe. The liver showed fatty degeneration. The brain showed slight bleeding. There were extensive hemorrhages into the mucous membranes. Generalized hemosiderosis was observed throughout the whole reticulo-endothelial system and in the liver cells. Brown iron free pigmentation of the skin was also observed. Rudimentary centers of blood formation with development of megakaryocytes were found in the lymph nodes and spleen. Areas of chronic inflammation were seen in the suprarenal medullae and in the interstitial tissue of the kidneys.

**Case 2 Sch. Willi.** The younger brother was born in 1923 and is now 25 years old. The course of his illness is shown in figure 4.

Past illnesses were whooping cough, measles, mumps and bronchitis during childhood and appendectomy at the age of 15.

The present illness started in November 1943 at the age of 20 with a cold which was followed by pneumonia of the left lower lobe while the patient was in military service. Following Cabazol therapy the pyrexia diminished but a low grade fever continued and a high sedimentation rate persisted with readings of 90 mm in the first hour and 105 mm in the second hour (Westergren). The hemoglobin at the onset of the illness was 86 per cent later dropping to 60 per cent.

Following blood transfusion therapy the hemoglobin was 86 per cent but later dropped and the anemia became even more severe. The disease continued to progress, with a hemorrhagic tendency always the most prominent feature, with especially bad bleeding following the extraction of a tooth. He continued to run a low grade fever with temporary bouts of higher pyrexia of unexplained origin. Occasional episodes of diarrhea which were resistant to therapy occurred. In 1945, the patient began to suffer from



FIG. 2. STERNAL PUNCTURE OF SCH FRANZ

(a and b) Microphotographic enlargement ( $\times 1000$ ) Clusters of plasma cells with central reticular cells

(c) From the same slide photograph from watercolor picture. Smaller basophilic stroma cells (plasmacytic, histiocytic and fibrocytes-like cells)

violent pain in the bones, and had intercurrent eosinophilic infiltration of the lungs and an attack of epidemic hepatitis. At times there was spontaneous improvement in his condition. The disease was resistant to all forms of therapy including sulfonamides, penicillin in large doses, iron and massive doses of vitamins. Temporary improvement could be brought about only by transfusions. He was given more than 70 transfusions totalling about 20 liters of blood.

Because of the failure of all other therapeutic measures splenectomy was carried out in September 1945. Following operation the hemoglobin increased to 70 per cent, the bleeding tendency ceased the

general condition improved and the weight increased. However, a few weeks after operation the anemia again increased with a recurrence of bleeding into the skin. Violent pains occurred in the bones of the legs and thighs, in the shoulder blades and the vertebral column. The skin of the legs gradually became

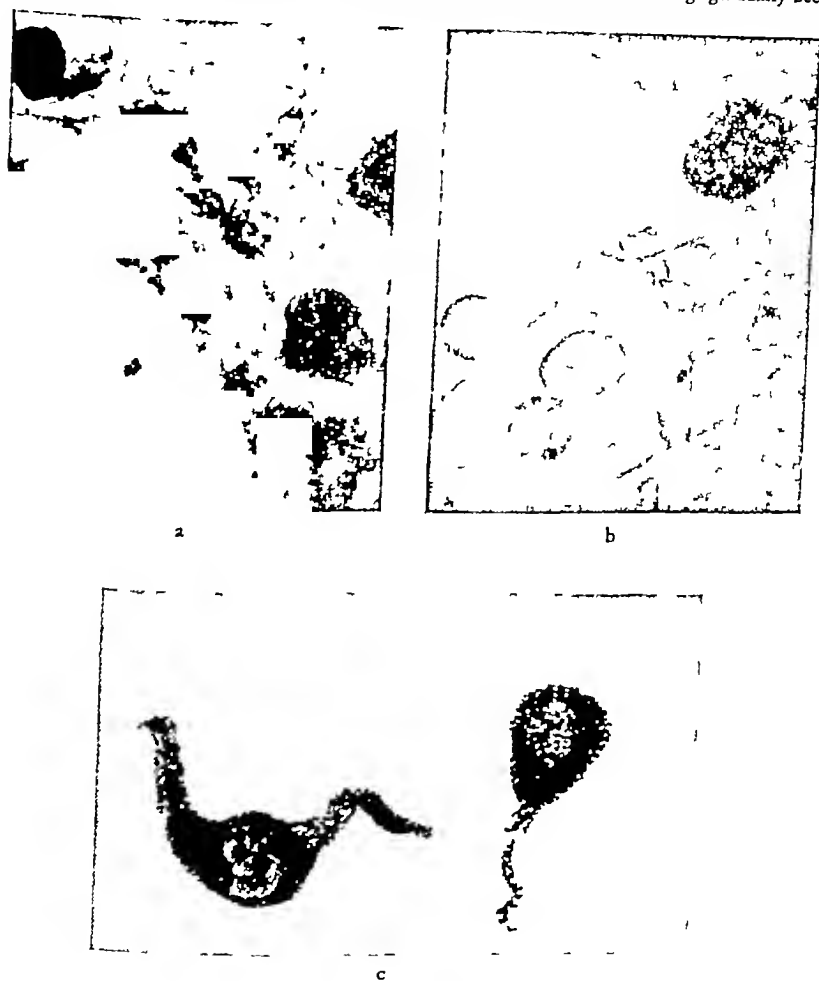


FIG. 3 STERNAL PUNCTURE OF SCH. FRANZ

(a and b) Microphotographic enlargement ( $\times 1000$ ) Various tissue mast-cells besides connective tissue and small lymphoid reticular cells

(c) Two isolated mast cells in aplastic anemia, photograph from a watercolor picture. Note the coarse eosinophilic granulation and the protoplasmic pseudopodia

greyish like smoke with dark pigmented spots. At the beginning of January 1946 the patient was given high altitude therapy in the Engadine. His clinical condition became stationary with frequent violent pains in the legs and pigmentation of the hands. The hemoglobin at this time was 65 per cent.

The clinical condition remained unchanged up to the end of April 1947. The patient had been able to do some light work for several months. He often complained of violent boring pain in the bones of legs

the cervical vertebrae, the shoulder blades and the bones of the jaw. After preparation by blood transfusions, 11 decayed teeth were extracted without any severe bleeding. Only rare bleeding into the skin and epistaxis had occurred and there had been no hematuria. The temperature had been only slightly elevated except during an influenzal infection. The hemoglobin on March 30, 1947, was 59 per cent. Liver and folic acid therapy were without effect.

Physical examinations done at various times during the illness showed striking pigmentation of the skin, especially around old scars, as well as brownish spots of pigmentation on the mucous membrane of the mouth. The skin was delicate and decidedly, smoke grey in color, particularly on the legs and thighs, and to some extent on the arms and body. In a few places some darker spots were noticed. It was observed that the patient had a slender skull and x rays revealed a thin skull with a small sella turcica. The structure of the body was somewhat asthenic and gave the impression of being slightly infantile. There were few hairs on the body, with hardly any beard growth and feminine genital hair distribution (fig. 3). He was found to be intellectually normal. No abnormalities were found by x ray in the pelvic bone, femur or humerus.

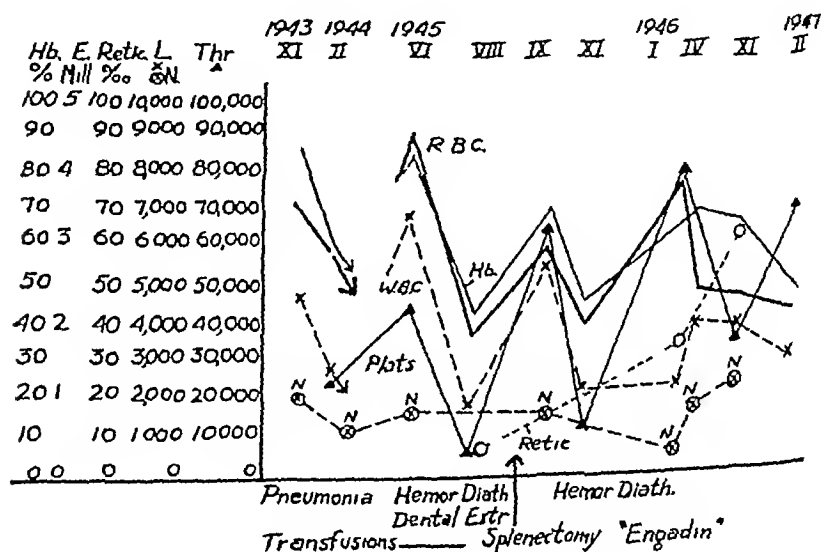


FIG. 4. COURSE OF THE BLOOD COUNTS IN SCH. WILLI

The urine gave a slightly positive test for urobilin and occasionally showed a few isolated red cells. Free hydrochloric acid was present in the stomach. The stools contained increased amounts of fats, but no increase of urobilin. X rays showed the stomach and intestines to be normal. The electrocardiogram showed deflection of the T wave in the second lead but was otherwise normal. The basal metabolic rate was +4 per cent.

Blood chemistry showed total proteins varying from 7.0 to 7.8 gm per 100 cc, cholesterol 147 to 173 mg per 100 cc, serum iron 171-215 gamma per 100 cc, calcium 9.5 mg per 100 cc, nonprotein nitrogen 27 mg per 100 cc, the uric acid 4.2 mg per cent and the bilirubin 0.3 mg per cent except during the attack of epidemic hepatitis when it rose to 6.2 mg per cent. The Takata Ara reaction was negative and the Weltman coagulation band was 0.2 (enlarged).

The Wassermann, Pitquet and Mantoux reactions were negative, and repeated blood cultures were also negative.

**Hematologic findings.** The hemoglobin varied between 59 and 65 per cent except after transfusions and a rise to 70 per cent following splenectomy. The red cell count varied from 2.4 to 3.0 million per cu mm.

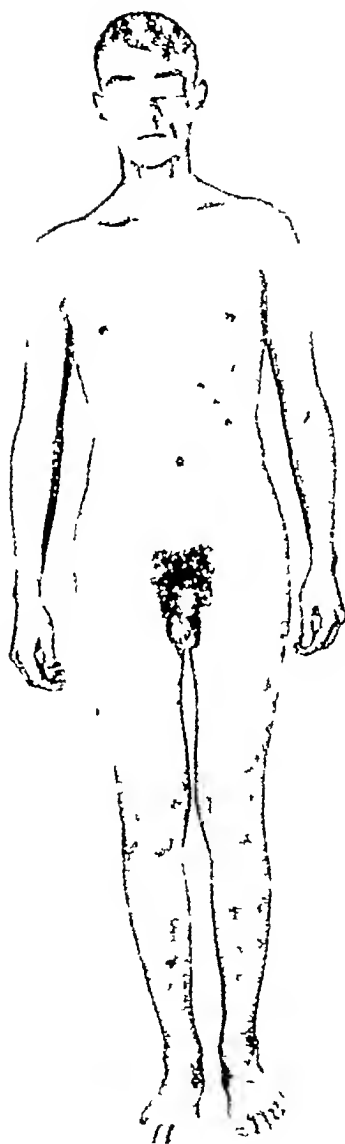


FIG. 5 SCH. WILLI

Note the slight infantile aspect, the feminine hair growth and the pigmentation of the skin, especially on the legs. Status after splenectomy.

and the color index from 1.0 to 1.3. The white cell count showed leukopenia, 2,800 to 4,500 except after transfusions and after splenectomy when it rose to 5,500. Neutrophils were 39.5 to 45.5 per cent.



FIG. 6. BLOOD PICTURE OF SCH. WILLI

Microphotographic enlargement ( $\times 1000$ ) Note the enormously developed anisocytosis in (a) macrocytic and microcytic (schistocytes) forms and in (b and c) the target cells

sinophils 0.5 to 5.0 per cent, basophils 0.0 to 0.5 per cent, monocytes 9 to 12 per cent, lymphocytes 40 to 57 per cent with a rise to 63 per cent following splenectomy and 1.5 per cent plasma cells were seen on one occasion. The platelets were markedly reduced 25 000 to 46 000 except immediately after splenectomy when they were 66 000. The reticulocytes were 1.1 to 2.3 per cent. Blood smears (fig. 6) showed

anisocytosis with very large and very small cells poikilocytosis, polychromasia, and even before splenectomy erythroblasts and Howell Jolly bodies were present

The bleeding time was 6 minutes with a temporary rise to 30 minutes in 1945. The prothrombin time was 40 per cent later 100 per cent. The coagulation time was 1 to 12 minutes. The osmotic fragility test showed initial hemolysis in 0.5 per cent NaCl and complete hemolysis in 0.1 per cent NaCl.

The sedimentation rate was persistently elevated. Early in the disease it was 45 mm in the first hour and 75 mm in the second hour by the Westergren method rising to 70 mm and 110 mm in the first and second hours respectively except for readings of 8 and 15 respectively during a remission in the anemia. In April 1946 the readings varied from 18/39 to 26/45 and in March 1947 the result was 28/47.

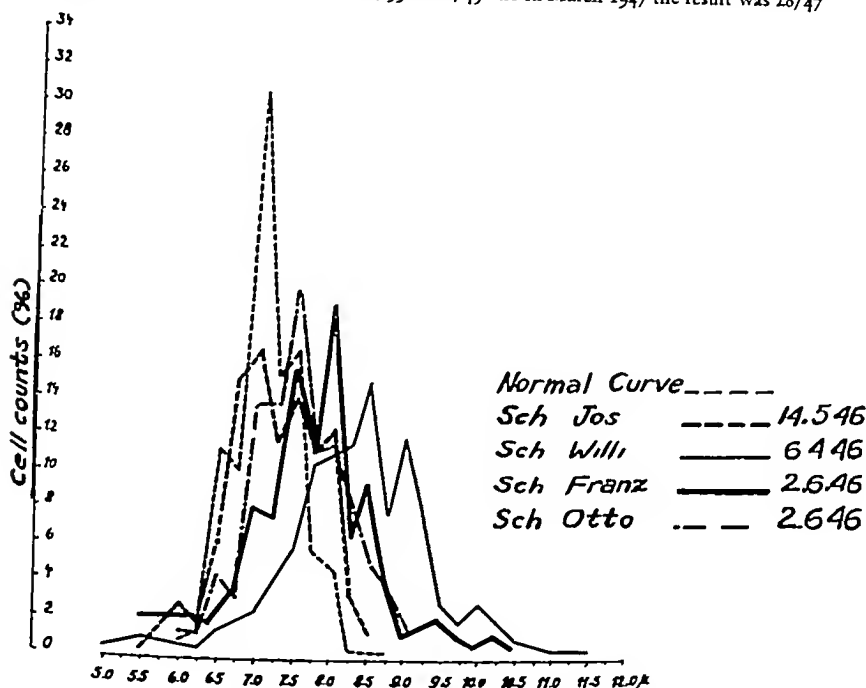


FIG. 7. PRICE JONES CURVE OF THE FOUR BROTHERS

Note in comparison with the normal curve the deflation and broadening of the curve especially toward the right, a tendency which is strongly marked in the still living patient, Sch Willi; in the deceased Sch Franz less remarkable and scarcely noticeable in the healthy brothers.

**Histologic findings.** The spleen histologically showed moderate thickening of the capsule and trabeculae as well as thickening and hyalinization of the intima of the follicular arteries. The pulp contained copious amounts of red cells with a moderate number of lymphocytes, some neutrophils and eosinophils, numerous hemosiderin-containing pulp cells and some plasma cells. The venous sinuses were also enlarged. A diagnosis of chronic splenomegaly and hemosiderosis was made.

Sections of a growth excised from the subcutis of the leg showed perivascular lymphatic infiltrations with some isolated polymorphs. Numerous hemosiderin-containing cells were found in the connective tissue as well as in the corium.

Sections of the bone marrow also showed numerous hemosiderin-containing macrophages and a few lymph follicles. The bone marrow was also studied by sternal puncture done seven times during the course of the disease. The corticalis was moderately hard. At first the marrow was rather abundant but later became scarce. The most consistent finding was an increase of the immature myelocytes and of big ba



toxic erythroblasts while megakaryocytes were seen rarely. In several punctures many reticular cells, especially larger and smaller plasmacytic forms were present. On one occasion tissue mast cells were remarkably abundant and they were seen in a few other instances especially in places where the bone marrow was thick.

*Family History* The maternal grandparents died at 70 and 84 years of age both of cancer of the stomach. The father is 56 years old. The mother is 53 years old and suffers from mild hypertension but is otherwise well. Of 8 paternal uncles and aunts one has tuberculosis and 2 suffer from chronic polyarthritis. Two brothers are living and well. There were twin sisters, one of whom was stillborn and the other died one hour after birth.

Of 45 relatives examined only 2 showed hematologic abnormalities. In 2 otherwise healthy brothers, with hemoglobins of 100 and 102 per cent and red cell counts of 4.8 and 5.0 million per cu. mm. respectively the Price Jones curves showed a tendency to widening of the base both to the macro- and microcytic sides (fig. 7). Their reticulocytes sometimes rose to 2.0 and 2.4 per cent and the serum iron concentrations were 140 and 165 gamma per 100 cc. Osmotic fragility tests showed initial hemolysis in 0.45 and 0.46 per cent NaCl respectively and complete hemolysis in 0.30 per cent NaCl in both.

The second patient and the parents were Rh positive.

## DISCUSSION

One of the notable features of the disease in these two brothers lies in their strikingly analogous clinical symptomatology. The features common to both cases may be listed as follows:

1 *Age of onset of symptoms* In the case of the elder brother, symptoms began when he was 24 years old, although signs were already present five years earlier. The younger brother became ill at the age of 20.

2 *Pigmentation* In both patients an abnormal pigmentation of the skin attracted attention, showing sometimes a brown, sometimes a more smoky grey color. Pigmentation was present in one brother even before other manifestations of the disease appeared, and in the other patient the degree of pigmentation was greater than could be accounted for by the hemorrhagic diathesis or by the numerous blood transfusions.

3 *Hemosiderosis* In both cases, histologic examination revealed an abnormally marked hemosiderosis in the reticulo-endothelial system.

4 *Pain in the bones* Both patients complained at times of violent pain in the bones.

5 *Panhemocytopenia* In both cases the entire bone marrow was affected from the very beginning, with resultant anemia, leukopenia and thrombocytopenia.

6 *Hematologic findings* Both patients had a hyperchromic type of anemia, with a color index between 1.1 and 1.4. The erythrocytes revealed unusually marked anisocytosis with large macrocytes and some abnormally small microcytes (so-called schistocytes). Furthermore, in both cases there was a tendency to poikilocytosis, occasional target cell formation and to an abnormal amount of polychromasia. The number of reticulocytes was almost constantly above normal in both patients. The serum bilirubin was normal, the Takata-Ara test negative and the Weltman coagulation band enlarged and the Wassermann test negative.

7 *The morphology of the bone marrow* At the beginning of the illness only the signs of maturation arrest were apparent. Later, hypoplasia of the marrow parenchyma appeared which progressed to almost complete aplasia of the marrow in the patient who died. Moreover, in both cases, striking changes were present in the

stroma There was marked increase of small as well as larger forms of plasmocytic reticular cells (plasmocytosis), constant increase of the fibrocytes (fibrosis), and in addition unusually exuberant growth of the so-called tissue mast cells (mastocytosis), with as many as 4 to 5 such cells per field in some areas

Additional features of the disease are as follows (a) The younger, still living patient showed certain signs not observed in his brother, namely slight infantilism with deficient hair growth, microcephaly, a small hypophysis and hypogenitalism (b) In one patient the osmotic fragility of the red cells was increased at the beginning of the illness, while it was normal at the beginning of the illness of the other (c) In one patient a few Howell-Jolly bodies and erythroblasts were seen in the peripheral blood even before splenectomy (d) The level of serum iron was continually high in one patient, but was not determined in the other (e) There have been no previous reports in the literature of the occurrence in adults of a similar familial form of panhemocytopenia accompanied by such striking pigmentation Many cases of familial anemia, agranulocytosis and panmyelophthisis have been reported, especially by Gaennslen and Huber<sup>1</sup> However, the clinical picture of the two patients reported here seems to bear more resemblance to the constitutional panmyelopathy of children, described first by Fanconi<sup>2</sup> in 1927 and known as anemia perniciosiformis constitutionalis, or the Fanconi syndrome This disease has also been described by Uehlinger,<sup>3</sup> Zellweger and Zollinger<sup>4</sup> and by Dameshek and associates<sup>5</sup> The condition is characterized by a refractory macrocytic anemia with leukopenia and thrombopenia, brown pigmentation of the skin, microcephaly, atrophy of the testes and a tendency to deformities of the skeleton

Hematologically, we are apparently dealing with the same anomaly in the patients reported here Furthermore, as reported in the disease in children, these patients showed pigmentation of the skin, due apparently chiefly to hemosiderosis The infantile features were less pronounced here, though they could be seen distinctly in one of the patients The less pronounced degree of these changes seems to be connected with the relatively late development of the disease, which set in after the completion of puberty in both patients

Unlike the known aplastic anemias which are either normochromic or show a tendency to macrocytosis, it is of considerable interest to find in these patients an unusually marked anisocytosis with, on the one hand, very large macrocytes, and on the other hand, very small microcytes (so-called schistocytes), as well as poikilocytosis and target cell formation The reticulocytes were increased up to 20 to 30 per cent, whereas they are usually lacking in typical cases of aplastic anemia Although there was little or no increase of bilirubin in the serum, and the urobilin elimination in the urine was insignificant, there were various other factors which indicated pathological hemoglobin metabolism One indication was increased hemolysis, suggested by the high concentration of serum iron, the abnormal osmotic fragility of the erythrocytes and the number of reticulocytes Another was the pathologic iron storage throughout the reticulo-endothelial system, as indicated by the hemosiderosis of the various organs \* The increase of

\* This may have been due at least in part to the effects of multiple transfusions It is curious that exogenous hemochromatosis seems to develop much more extensively in cases of hypoplastic anemia than in some other cases of anemia given numerous transfusions *Editor*

hemolysis might be explained by assuming that a more exact balance of hemoglobin metabolism existed

The pathologic functioning of the reticulo-endothelial system in the two patients studied manifested itself not only in the generalized hemosiderosis, but also in changes in the bone marrow. As mentioned above, the changes in the reticulum and in the stroma of the marrow were especially remarkable, consisting of marked growth of the reticular cells, especially of the large and small plasmocytes, of the tissue mast cells and of the fibrocytes. These pathologic changes can be summed up with the designation reticulo-fibrosis of the bone marrow. The changes in the stroma seem to represent the primary disturbance, the first changes being plasmocytosis and mastocytosis. This results in maturation arrest of the normal marrow parenchyma which follows as the next stage of the process. With the evolution of the disease there ensues a kind of cicatrization process, an increase of the fibrosis with a gradual destruction of myeloid tissue and marrow atrophy is a still later stage of the process.

At present no definite answer can be given to the question of the physiopathologic importance of the enormous increase of the plasmocytes and mastocytes. However, it is known that both cellular forms should be classified in the reticulo-histiocytic system and that they belong to the so-called active mesenchyma. The plasma cells undoubtedly play an important part in the formation of globulin, particularly of gamma globulin, and hence in the development of antibodies. Thus, a relationship between plasma cells and certain immunity reactions appears to be important. On the other hand, the mastocytes, which show a genetic relation to heparin and amyloid, presumably have some connection with anaphylactic processes. \* It is theoretically possible that these particular plasmocytic and mastocytic changes of the bone marrow are an expression of an *anaphylactic-allergic process* of the bone marrow. In the light of these facts, it is noteworthy that in both patients the whole clinical picture developed in connection with an infectious disease (poliomyelitis and pneumonia respectively). Such a pathologic reaction of the reticulo-histiocytic system not only explains the primary reaction of the stroma of the bone marrow with a tendency to fibrosis of the marrow, but also accounts for the abnormal blood picture.\*

Other pathologic conditions of the reticulum or mesenchyma are known to be accompanied by even greater disturbances of the blood picture. This is true especially of osteosclerosis and osteomyelosclerosis, where the principal disturbances originate in the osteogenic reticulum, and in Cooley's anemia, where it is the disturbance of the myelogenic reticulum which seems to be chiefly responsible for the disturbances in the formation of blood. In these blood diseases, similar morphologic changes of the erythrocytes are found, namely marked aniso-, micro-, macro-, poikilocytosis, and target cells. These changes are much more pronounced in Cooley's anemia. In both these diseases there is not only a disturbance in the formation of blood but also a disturbance in the development of the bones. One

\* We have found tissue mast cells in the bone marrow in but a dozen cases and only in hypoplastic and aplastic anemias of various etiology (benzol poisoning, leukemia myeloma, infections and idiopathic forms).\*

disturbance is not the consequence of the other, but pathologic changes occur in both organs from the beginning. In Cooley's anemia, however, the pathologic blood formation is more striking, and in osteosclerosis the pathologic bone formation dominates the clinical picture.

It is not difficult to explain generalized hemosiderosis and pigmentation of the skin and mucous membranes as a consequence of pathologic functioning of the reticulo-histiocytic system. Abnormal hemolysins or agglutinins were not detectable in the two patients reported here. The parents and the patient who is still living are all Rh+.

#### SUMMARY

An account is given of a similar and hitherto unknown clinical-hematologic syndrome in two adult brothers with marked hemorrhagic diathesis, diffuse pigmentation of the skin, violent pain in the bones and panhemocytopenia. In the younger brother, there is also a certain degree of infantilism. The elder brother, died with all the symptoms of an intensive aplastic anemia, in the younger brother, the condition was stabilized after splenectomy. The blood picture in both patients was characterized by a hyperchromic anemia with remarkable micro- and macrocytosis, and an increased number of reticulocytes. In the younger brother, increased fragility of the red blood cells and an elevated serum iron content were observed. In both cases, an unusual increase of the plasmocytic and reticular cells and of the tissue mast cells was noticed in the bone marrow and, in the final stages of the disease, the marrow showed marked fibrosis.

The disease is believed to be a variety, in adults, of the syndrome first described by Fanconi as a constitutional panmyelopathy occurring in children. The illness is the result of a hereditary pathologic reaction of the reticulo-histiocytic system and seems to have been caused by an anaphylactic-allergic phenomenon. The possibility is discussed that genetic connections may exist between this condition and other diseases, such as certain osteoscleroses and Cooley's anemia, which are characterized by simultaneous disturbances of the bone and bone marrow and by a similar blood morphology.

#### REFERENCES

- <sup>1</sup>HUBER H. Stammbaumentersuchungen bei Panmyelophthisekranken. *Klin. Wchnschr.* 18: 1145, 1939.
- <sup>2</sup>FANCONI G. Familiäre infantile perniziösartige Anämie. *Jahrb. f. Kinderk.* 117: 257, 1927.
- <sup>3</sup>UEHLINGER E. Konstit. infantile (perniziösartige) Anämie. *Klin. Wchnschr.* 1501: 1929.
- <sup>4</sup>ZELLWEGER H. AND ZOLLINGER, H. U. Ueber einen sporadischen Fall von konstitut. infantiler Panmyelopathie Fanconi. *Helv. Paed. Acta* 1: 382, 1940.
- <sup>5</sup>ESTREN, S. SUESS J. F. AND DAMESHEK W. Congenital hypoplastic anemia with multiple developmental defects (Fanconi syndrome). *Blood* 2: 85, 1947.
- <sup>6</sup>ROHR, K. Das menschliche Knochenmark. Leipzig: Thieme, 1940.

## EXTRAMEDULLARY BLOOD PRODUCTION

By CLAUD MUNK PLUM, PH D

IN MAMMALS, throughout postnatal life, erythrocytes and granulocytes are normally produced only in the bone marrow, while the third type of circulating blood cell, the lymphocyte, is produced chiefly in the lymph nodes and the spleen, and only to a relative small extent in the bone marrow. During fetal life, blood formation occurs to a large extent in organs other than the bone marrow. It is well known that the liver and spleen take part in fetal blood formation and that this function ceases at birth, but no one seems to have investigated the function of the mammary and prostate glands with regard to existence and duration of hematopoietic activity within them. The observations to be reported at this time attempt to correlate the occurrence of extramedullary hematopoiesis in the mammary glands and the prostate, with the hematopoietic functions of the liver and the spleen.

Extramedullary blood formation by the newborn has been studied extensively in the past few years.<sup>1 7 10 11 12</sup> Bertelsen<sup>1</sup> investigated the origin of the erythrocytes during the last fetal months and the first days of extrauterine life, studying especially the liver, spleen and the thymus gland. Schlachta<sup>17</sup> found extramedullary foci in the prostate and the suprarenal glands, Block<sup>2</sup> in the kidneys and the renal pelvis, Gruber<sup>8</sup> in the mammary glands, and Weil<sup>10</sup> in the skin of the soles of the feet. The observations by Marchand and Lohlein<sup>14</sup> of extramedullary foci in the greater omentum and the sole of the foot laid the foundation for all the more recent investigations of the problems of extramedullary blood formation. These authors found that the perivascular cells, perhaps similar to Saxer's primitive histiocyte,<sup>16</sup> were the origin of the great stabformed types of basophilic and eosinophilic granulated cells and of the erythroblasts. The occurrence of the erythroblast in the greater omentum, however, was denied by Seifert.<sup>18</sup>

Weil's description of extramedullary hematopoiesis in the sole of the foot in human beings was based upon detailed investigations in only 4 cases.<sup>20</sup> The cells of the hematopoietic foci were found about the sweat tubules or in the adipose tissue. From his investigations the author concluded that the foci of blood formation always occurred in relation to the sudorific glands or to glands which might be a modification of sudorific glands, e.g., mammary glands. Weil's studies were continued by Dieterich,<sup>5</sup> who especially observed the mammary glands and the skin of the hand, foot, head and axilla. Dieterich's observations were based on a relatively large number of cases: 4 fetuses aged 2 to 6 months, 14 newborn individuals, 10 children aged 8 days to 3 years, and 11 adults. He found, in 10 newborn infants, that the pedal extramedullary foci were limited to the sole of the foot, no foci being present in the dorsum of the foot. This observation agreed with Weil's inference concerning the occurrence of hematopoietic tissue in relation to sudorific glands.

From the Department of Pathological Anatomy University of Copenhagen, Denmark

In the mammary glands, Dieterich found hematopoietic foci which greatly resembled bone marrow. These foci contained mainly cells of the myeloid and the lymphoid series, erythroid elements being rarely found (in one 11 year old child, however, large numbers of erythroblasts were present). Such observations help to confirm the theory advanced by Morawitz and Rehn (quoted by Dieterich) concerning a reciprocal action between the leukocytic and erythroid system. Extramedullary foci were rarely found in the skin of the hand, and never in the skin of the back, head or axilla (Dieterich<sup>6</sup>). Extramedullary foci were never found in the normal adults.

As mentioned previously, the formation of erythrocytes and granulocytes normally takes place in the bone marrow, but this normal formation may be supplemented in pathologic cases by extramedullary blood formation. Such ectopic hematopoiesis frequently takes place in the spleen and the lymph nodes, less frequently in the liver, and only rarely in the suprarenal glands, kidney, cartilages, the broad ligaments, and scattered throughout the adipose tissue of the organism. In general, the sites of extramedullary blood formation which are found normally in embryonic and fetal life, are also the sites in which the phenomenon occurs under pathologic conditions in the infant and adult.

Extramedullary hematopoiesis occurs under various pathologic conditions in adult mammals. Recorded cases refer chiefly to man. In infants and young children, extramedullary hematopoiesis is often found in association with severe anemia.<sup>3</sup> Various authors<sup>4-19</sup> have reviewed the recent literature on ectopic blood formation in erythroblastosis fetalis. In pernicious anemia during relapse, extramedullary hematopoietic foci are regularly found in the spleen and liver.<sup>13-16</sup> In macrocytic anemias, especially those associated with liver disease, such foci are frequently found in the spleen.<sup>21</sup> Extramedullary foci occur in osteosclerosis,<sup>6</sup> in invasion of the bone marrow due to various causes,<sup>3-10,12</sup> and in Hodgkin's disease,<sup>21</sup> even when the anemia is not very severe. Ectopic blood formation has been described in erythremia,<sup>21</sup> hemolytic jaundice,<sup>9</sup> and leukemia.<sup>12</sup> Tumors of heterotopic bone marrow have been observed in adipose tissue of patients with anemia in severe sepsis.<sup>21</sup> Extramedullary hematopoiesis has been produced experimentally by repeated bleeding and by chronic poisoning with blood-destroying substances.<sup>3</sup>

In general, the extramedullary foci may be composed of erythroid elements, myeloid elements, megakaryocytes, or of all three types of cells. The ectopic hematopoiesis is often interpreted as a compensatory phenomenon, evidence for such a theory being, among other things, the readiness with which such a change occurs in infants and young children, in whom the bone marrow has little or no room for expansion.

#### PRESENT INVESTIGATIONS

The present report deals with the results of the examination of a total of 94 individuals, including 79 fetuses from 6 months of age to birth, and 15 children and adults from 1 day to 25 years of age. All fetuses with erythroblastosis fetalis were omitted, and only normal material was used. The material was collected from the Department of Pathological Anatomy, University of Copenhagen.

TABLE 1 — Extramedullary Hematopoietic Foci in Various Organs of 94 Individuals Including 79 Sexu and 15 Postfetal Subjects

Age	Case no	Sex	Weight	Length	Liver		Spleen				Breast	Pro-state	Planta pedis	Supra-renal glands
					Lob foci	Port foci	Pulp		Follicles					
					(1)	(2)	Ery t	Myeloc	Ery t	Myeloc				
			Gm	cm			(3)	(4)	(5)	(6)				
6 months	24	f	750	30	138 2	1 9	33 9	0 3	0 2	0 1	++			
	46	m	570	27	203 5	2 4	49 6	0 4	0 2	0 1	(+)	++		
7 months	190	f	1000	39	80 3	1 2	23 2	0 4	0 3	0 2	++		+	
	218	f	950	36	102 5	2 3	36 0	0 4	0 3	0 1	++		+	+
	238	f	1100	39	59 6	1 9	29 5	0 3	0 2	0 2	+++		+	
	293	f	770	34	132 3	2 0	26 8	0 3	0 3	0 3	++		o	
	257	f	900	39	77 2	1 6	19 3	0 2	0 2	0 1	+++		o	+
	200	f	1050	40	62 5	2 1	27 7	0 3	0 2	0 1	++		+	
	201	f	950	39	100 0	0 9	15 2	0 2	0 3	0 1	+++		o	
	311	f	1300	36	26 2	2 7	12 9	0 2	0 2	0 1	+++		+	+
	243	f	970	34	90 9	0 6	33 2	0 3	0 3	0 1	++		(+)	+
	161	m	1100	42	92 -	0 9	26 2	0 3	0 -	0 1	+	++	+	+
	174	m	800	32	121 9	2 2	39 4	0 3	0 -	0 1	+++	+++	+	
	175	m	1100	38	79 5	1 3	22 3	0 2	0 1	0	+	++	(+)	+
	240	m	950	44	139 0	1 3	36 5	0 4	0 2	0 1	+	++	o	+
	241	m	800	43	152 0	1 0	40 7	0 4	0 4	0 2	+		o	+
	27	m	1800	39	31 0	0 9	13 6	0 2	0 1	0	(+)	+		
	47	m	1300	37	56 2	1 2	16 2	0 2	0 1	0	o	(+)		
8 months	153	f	1500	43	33 8	1 2	28 6	6 4	0 2	0	+		o	o
	157	f	2150	40	89 6	0 8	10 4	0 2	0 1	0 1	++		+	+
	171	f	1100	38	77 4	0 6	19 6	0 3	0 2	0 1	+++		+	(+)
	173	f	1700	42	35 7	0 7	14 2	0 2	0 1	0	++		+	
	230	f	2100	45	29 4	0 4	21 4	0 1	0 3	0 2	++		+	(+)
	288	f	2300	44	18 2	0 4	10 2	0 2	0 1	0	++			
	312	f	2100	43	22 4	0 8	11 2	0 1	0 2	0 1	++			
	162	m	900	40	131 0	2 8	41 9	0 4	0 3	0 2	++	++	+	(+)
	167	m	2450	50	16 2	0 8	10 2	0 2	0 2	0	(+)	o	(+)	(+)
	169	m	1350	41	90 2	1 3	17 2	0 3	0 2	0 1	+	+	(+)	(+)
	237	m	1400	40	71 8	1 9	23 7	0 3	0 2	0 1	(+)	++	(+)	(+)
	242	m	1750	43	43 2	1 2	33 4	0 4	0 1	0	+	++	+	(+)
	13	m	1900	46	21 4	1 0	11 1	0 1	0	0	+	++	+	
	28	m	1650	42	80 3	1 7	33 1	0 2	0 1	0	(+)	(+)	o	
9 months	151	f	2700	51	14 7	0 8	7 8	0 7	0 1	0 1	+		(+)	o
	172	f	3200	50	2 4	0 6	2 9	0 1	0	0	+++		+	o
	183	f	1700	45	11 2	0 4	6 4	0 6	0 2	0 2	+			
	204	f	1550	43	53 7	2 2	12 4	0 2	0 1	0 1	++		(+)	(+)
	212	f	2000	46	16 2	0 9	5 6	0 2	0 3	0	+			
	230	f	2150	45	5 8	0 6	3 8	0 6	0 1	0 1	(+)			
	239	f	3400	54	11 9	0 3	6 9	0	0 1	0	+		(+)	(+)
	250	f	1450	43	33 2	2 6	10 2	0 5	0 1	0	++		(+)	(+)
	310	f	2250	46	6 3	0 8	6 7	0 5	0 1	0	++			
33	f	2800	52	22 2	0 8	13 7	0 2	0 1	0	o				

TABLE 1—Continued

TABLE 1—Continued

Age	Case no.	Sex	Weight	Length	Liver		Spleen				Breast	Prostate	Planta pedis	Supra renal glands
					Lob foci	Port foci	Pulp		Follicles					
							Eryt	Myeloc	Eryt	Myeloc				
			Gm	cm	(1)	(2)	(3)	(4)	(5)	(6)				
9 months	163	m	1400	43	37 7	1 1	14 2	0 8	0 2	0 1	o	+	(+)	o
	191	m	2400	46	26 3	1 7	9 5	0 2	0 1	0 1	o	+	(+)	o
	244	m	1550	49	46 8	2 0	18 9	1 2	0 2	0 1	o	+	(+)	o
	247	m	1550	43	18 2	1 0	9 2	0 1	0	0	o	+	(+)	o
	270	m	1200	45	11 4	0 4	7 3	0	0	0	o	+	(+)	o
	273	m	1200	40	63 5	2 2	13 5	0 4	0 1	0 1	o	+	(+)	o
	274	m	1150	39	48 2	1 7	12 2	0 4	0 2	0 1	o	+	(+)	o
	275	m	2600	47	8 0	0 2	4 3	0 2	0	0	o	+	(+)	o
	12	m	1300	46	18 2	0 5	16 2	0 2	0 2	0 1	o	+	(+)	o
	14	m	2400	48	61 4	1 8	27 2	0 3	0 2	0 1	o	+	(+)	o
	32	m	2000	43	70 2	2 4	26 1	0 4	0 3	0 2	o	+	(+)	o
	39	m	2650	52	19 7	0 7	12 4	0 1	0	0	o	+	(+)	o
Full grown	154	f	3500	54	5 8	0 2	9 2	0 1	0 1	0 1	+	+	+	+
	182	f	2500	52	11 4	1 2	6 9	0 2	0 4	0 1	+	+	+	+
	179	f	2850	52	31 6	0 9	16 7	0 4	0 1	0	+	+	+	+
	189	f	3350	53	47 8	2 9	22 1	0 3	0 6	0 2	+	+	+	+
	221	f	2400	50	8 2	0 6	7 9	0 1	0 2	0	+	+	+	+
	239	f	3400	54	6 6	0 8	7 2	0	0 2	0 2	o	+	+	+
	245	f	3100	51	35 8	1 4	16 3	0 1	0 1	0 1	o	+	+	+
	208	f	2900	52	49 8	0 8	11 2	0	0 3	0 1	+	+	+	+
	268	f	3100	50	18 2	1 2	9 2	0 3	0 1	0	+	+	+	+
	278	f	3200	51	9 4	0 4	7 2	0 2	0	0 1	+	+	+	+
	287	f	2550	50	11 4	1 0	8 2	0	0 1	0	+	+	+	+
	160	m	2500	48	7 6	0 6	2 3	0	0	0	+	+	+	+
	164	m	3200	50	10 1	1 2	4 3	0	0 1	0	+	+	+	+
	168	m	3500	54	56 6	2 4	12 3	0 3	0 2	0 1	+	+	+	+
	178	m	3500	50	23 8	0 8	10 6	0 1	0 2	0 1	+	+	+	+
	188	m	3350	50	16 2	0 8	3 4	0	0 1	0 1	+	+	+	+
	203	m	1800	50	13 7	0 8	5 9	0 1	0	0	+	+	+	+
	246	m	3650	53	0 4	0 2	1 3	0	0	0	+	+	+	+
	248	m	3400	52	26 5	0 8	9 8	0 2	0	0	+	+	+	+
	256	m	3600	53	11 5	0 9	11 3	0 1	0 2	0	+	+	+	+
267	m	3700	55	33 2	1 3	14 5	0 1	0 1	0	+	+	+	+	
276	m	3500	52	21 6	1 4	13 2	0 5	0 4	0 3	+	+	+	+	
285	m	3100	55	13 5	1 7	5 6	0 3	0 1	0 1	+	+	+	+	
11	m	4500	57	1 6	0 2	1 2	0	0	0	+	+	+	+	
23	m	3300	54	3 5	0 4	2 3	0	0	0	+	+	+	+	
1 day	277	f		1 4	0 2					+		o	o	
1 day	283	f		0 6	o					+		o	o	
1 day	295	f		1 2	0 2					+		o	o	
26 days	319	f		0 8	0 1					+		o	o	
95 days	278	f		0 5	0 2					+		o	o	



TABLE 1—Continued

Age	Case no	Sex	Weight	Length	Liver		Spleen				Breast	Prostate	Plantar pedis	Supra-renal glands
					Lob foci	Port foci	Pulp		Follicles					
					(1)	(2)	Eryt	Myeloc	Eryt.	Myeloc				
			Gm	cm			(3)	(4)	(5)	(6)				
1 day	437	m			0 9	0 2					o	(+)	(+)	o
11 days	339	m			2 1	o					o			
26 days	436	m			0 5	o					o	(+)	o	(+)
23 days	296	m			0 7	0 3					o			
42 days	291	m			0 3	0 2					o	(+)	(+)	o
5 months	393	m			0 2	0 1					o			
23 months	430	m			o	0 1					o			
6½ yr	396	m			0 1	o					o			
23 yr	206	m			o	o					o			
26 yr	203	m			o	o					o			

1) Average of 10 field of vision—lobular foci

2) Average of 10 field of vision—portal foci

3) Average of 10 field of vision—erythroblasts in the red pulp

4) Average of 10 field of vision—myelocytes in the red pulp

5) Erythroblasts/follicle

6) Myelocytes/follicle.

+++ Hematopoietic foci in each of ten field of vision

++ Hematopoietic foci in more than 67% of the fields of vision

+ Hematopoietic foci in more than 33% of the fields of vision

(+) Hematopoietic foci in less than 33%, but more than 0% of ten field of vision.

Blank space means no observation made in this case

The organs examined were as follows: mammary glands, liver, spleen, and, in some cases, the suprarenal glands and the soles of the feet. After removal from the body, the organs were fixed in 4 per cent formalin in 0.9 per cent sodium chloride for two days, and then were treated as usual for embedding in paraffin. For each organ 10–12 cuts were taken with intervals of 30  $\mu$ . The 5–6  $\mu$  slides were stained with three different dyes as follows: (1) hematoxylin-eosin, (2) van Gieson Hansen, and (3) May-Grunwald-Giemsa. The sections were examined under the microscope, and a notation made of the presence or absence of foci of hematopoiesis in examination of a number of fields. The particular magnifications used are given below.

As a hematopoietic foci was counted all crowding of cells, which belongs to the hematopoiesis, the cells are usually in different stages of the development. The number of cells varies.

### Results

The results of these investigations are listed in detail in table 1. Certain particulars are listed in tables 2 and 3.

*Liver* It would have been of interest to obtain a measure of the amount of hematopoiesis in the liver, but it was possible to obtain only a relative measure. The

average of foci found per microscopic field using Leitz objective 3-ocular 8, was recorded in the tables. In the liver, hematopoiesis takes place partly within the

TABLE 2.—*Number of Hematopoietic Foci in the Liver of Fetuses of Various Ages (The numbers in parentheses are from Bertelsen<sup>1</sup>)*

Lobular foci									
Maximum	152 0	(160 0)	131 1	(144 3)	70 2	(92 5)	56 6	(49 8)	
Minimum	26 2	(53 7)	16 2	(1 4)	2 4	(2 9)	0 4	(0 5)	
Average	87 7	(97 5)	54 3	(66 2)	27 6	(37 2)	19 0	(24 2)	
Portal foci									
Maximum	2 7	(2 4)	2 8	(3 0)	2 6	(2 4)	2 9	(3 0)	
Minimum	0 6	(0 6)	0 4	(0 7)	0 2	(0 2)	0 2	(0 5)	
Average	1 50	(1 48)	1 11	(1 36)	1 17	(1 18)	1 00	(1 34)	
Age	7 months		8 months		9 months		Full grown		

TABLE 3.—*Number of Hematopoietic Foci in the Spleen of Fetuses of Various Ages (The numbers in parentheses are from Bertelsen<sup>1</sup>)*

Erythroblasts in pulp									
Maximum	40 7	(46 7)	41 9	(32 0)	27 2	(26 9)	22 1	(33 9)	
Minimum	12 9	(9 1)	10 2	(6 2)	2 9	(4 2)	1 2	(2 5)	
Average	26 2	(23 6)	20 4	(18 9)	11 5	(14 6)	8 8	(12 2)	
Erythroblasts in follicles									
Maximum	0 4	(0 8)	0 3	(0 7)	0 3	(0 4)	0 6	(0 4)	
Minimum	0 1	(0)	0	(0)	0	(0)	0	(0)	
Average	0 25	(0 26)	0 17	(0 18)	0 12	(0 13)	0 14	(0 16)	
Myelocytes in pulp									
Maximum	0 4	(1 2)	0 4	(0 9)	1 2	(0 6)	0 5	(0 8)	
Minimum	0 2	(0)	0 1	(0)	0	(0)	0	(0)	
Average	0 24	(0 36)	0 24	(0 27)	0 35	(0 25)	0 15	(0 23)	
Myelocytes in follicles									
Maximum	0 3	(0 5)	0 2	(0 4)	0 2	(0 3)	0 -	(0 5)	
Minimum	0	(0)	0	(0)	0	(0)	0	(0)	
Average	0 11	(0 09)	0 07	(0 08)	0 07	(0 09)	0 06	(0 08)	
Age	7 months		8 months		9 months		Full grown		

lobes (lobular) and partly in the periportal connective tissue (portal) so that it was necessary to make a differentiation between these two groups

The numbers of foci in the livers of fetuses of various ages have been retabulated in table 2. We give here only the maximum and the minimum and the average of the numbers given in table 1

The number of such foci shows a progressive decline with increase in age, and during the last month of pregnancy the amount of hematopoiesis is markedly decreased, as seen in table 2.

*Spleen* In the spleen, the erythroblasts and the myelocytes were counted in the pulp and the follicles, averaging the number found in 10 microscopic fields using Leitz objective 1/12-ocular 8. The results are tabulated in table 3. It will be seen that erythropoiesis in the spleen still takes place at the end of fetal life.

*Mammary glands* In sections of the mammary glands, twenty fields of vision were employed (Leitz objective 3-ocular 8), and the amount of hematopoiesis listed from zero (0) to 3 plus (+++), as explained in table 1.

Although the material was small, it allowed the conclusion that the hematopoiesis was maintained to greater extent in the female than in the male fetuses.

These results agree with earlier investigations<sup>4, 10</sup> that the extramedullary foci occur in relation to the sudorific glands or their modifications, e. g., the mammary glands.

*Prostate* In the prostate, extramedullary hematopoiesis was observed in anatomic relation to the lobules, but the number of foci was not large. The findings confirmed the observations of Schlachta<sup>17</sup> that it is normal to find extramedullary foci here. The same seems to hold true for the mammary glands.

*The sole of the foot* Only a small part of the material was used for these investigations. Here there are small numbers of foci, almost always in relation to the sudorific glands, rarely in the adipose tissue.

*The suprarenal glands* Only a few cases were examined. In them, the extramedullary foci were very small and few in number.

#### COMMENT

These observations on the rather persistent extramedullary hematopoiesis in the mammary and prostatic glands seems to indicate that the immature blood cells find similar conditions in the bone marrow and the stroma of the gland in question. If this is so, an explanation may be forthcoming why malignant epithelial tumors arising in the breast and the prostate metastasize to the bone (in particular, to bone containing active blood-forming marrow). In this connection, it is of interest that extramedullary hematopoietic foci are present during fetal life in the lungs and the kidneys too, and that bronchogenic carcinoma and hypernephroma also very often metastasize to bones.

These statements are offered only as reflections. Further investigations along these lines are in progress.

#### SUMMARY

Investigations of extramedullary foci of hematopoieses demonstrate that, in human beings, the extramedullary hematopoiesis in the fetus at term is more pronounced in the mammary glands of the female than in those of the male. The author suggests the possibility that there may be a connection between the location of the foci, and the liability of metastasis in cancer, especially in mammary and prostatic cancer.

## ACKNOWLEDGMENT

The work was supported by a grant from King Christian the Tenth foundation and the Carlsberg Foundation. I bring my sincere thanks to both these foundations.

## REFERENCES

- <sup>1</sup> BERTelsen, A. Beiträge zur Frage der normalen Genese der Blutzellen. Thesis. Copenhagen, 1938.
- <sup>2</sup> Bloch, R. Virchow's Arch. 228: 285, 1920.
- <sup>3</sup> BRANNAN, D. Bull. Johns Hopkins Hosp. 41: 104, 1927.
- <sup>4</sup> DIAMOND, L. K. ET AL. J. Pediat. 1: 269, 1932.
- <sup>5</sup> DIETERICH, H. Arch. f. klin. Chirurg. 134: 166, 1925.
- <sup>6</sup> DONHAUSER, J. L. J. Exper. Med. 10: 559, 1908.
- <sup>7</sup> GILMOUR, J. R. J. Path. & Bact. 102: 25, 1941.
- <sup>8</sup> GRUBER, G. B. Ztschr. f. Kinderh. 30: 336, 1921.
- <sup>9</sup> HARTFALL, S. J. ET AL. J. Path. & Bact. 377: 455, 1933.
- <sup>10</sup> JORDAN, H. E. Arch. Path. 18: 1, 1934.
- <sup>11</sup> ———. Anat. Rec. 59: 461, 1934.
- <sup>12</sup> ———. Physiol. Rev. 22: 375, 1942.
- <sup>13</sup> LYALL, A. J. Path. & Bact. 41: 469, 1935.
- <sup>14</sup> MARCHAND, F. U. ET AL. Ztschr. f. allg. Path. & Pharm. 24: 951, 1913.
- <sup>15</sup> MEYER, E. ET AL. Verhandl. d. deutsch. path. Gesellsch. 9: 224, 1905.
- <sup>16</sup> SAXER, A. Anat. Hefte 1: 349, 1898.
- <sup>17</sup> SCHLACHTA, J. Arch. f. mikr. Anat. & Entw. 64: 405, 1904.
- <sup>18</sup> SEIFERT, Arch. f. klin. Chirurg. 123.
- <sup>19</sup> STRONG, R. A. ET AL. J. Pediat. 15: 658, 1939.
- <sup>20</sup> WEILL, I. Ztschr. f. Kinderh. 35: 19-3.
- <sup>21</sup> WINTROBE, M. M. Arch. Int. Med. 57: 289, 1936.

deficiency Table 1 shows the differences in body weight and white cell phagocytic activity of rats adapted to various graded vitamin deficiencies

TABLE 1—*Rat White Cell Phagocytosis in Graded Vitamin Deficiencies*

Group	Amount of vitamin per kilo of diet	At 68 F		At 90-91 F and 60-70% R. H	
		Aver body weight at end of period	Number of bacteria ingested in 4 min	Aver body weight at end of period	Number of bacteria ingested in 4 min.
Thiamin deficient rats tested after 4 weeks on diets					
		gm		gm	
1	0.6 mg	89	3.40 $\pm$ 0.1	63	2.63 $\pm$ 0.16
2	1.0 mg	146	3.66 $\pm$ 0.22	87	5.27 $\pm$ 0.23
3	2.0 mg	154	7.51 $\pm$ 0.7	124	7.40 $\pm$ 0.24
Riboflavin-deficient rats tested after 7 weeks on diet*					
1	0.0 mg	71	5.70 $\pm$ 0.27	81	3.81 $\pm$ 0.21
2	1.0 mg	165	8.13 $\pm$ 0.25	181	4.51 $\pm$ 0.24
3	2.0 mg	182	9.37 $\pm$ 0.28	216	7.54 $\pm$ 0.25
4	4.0 mg	196	12.00 $\pm$ 0.30	227	11.48 $\pm$ 0.51
Pyridoxine deficient rats tested after 7 weeks on diets*					
1	0.5 mg	129	4.95 $\pm$ 0.40	167	7.18 $\pm$ 0.27
2	1.0 mg	184	6.82 $\pm$ 0.36	192	9.23 $\pm$ 0.29
3	2.0 mg	204	8.60 $\pm$ 0.29	196	10.83 $\pm$ 0.32
4	4.0 mg	217	13.56 $\pm$ 0.42	202	14.13 $\pm$ 0.41
Pantothenic acid-deficient rats tested after 7 weeks on diets					
1	0.5 mg	76	2.43 $\pm$ 0.16	100	2.55 $\pm$ 0.21
2	1.0 mg	114	3.63 $\pm$ 0.21	89	3.78 $\pm$ 0.22
3	3.0 mg	151	4.58 $\pm$ 0.22	161	6.83 $\pm$ 0.21
4	6.0 mg	198	5.89 $\pm$ 0.27	149	5.92 $\pm$ 0.27
Choline deficient rats, tested after 6 weeks on diets					
1	0.0 Gm			147	3.08 $\pm$ 0.25
2	0.2 Gm	180	2.47 $\pm$ 0.15		
3	0.4 Gm	178	4.88 $\pm$ 0.27		
4	0.75 Gm	192	6.53 $\pm$ 0.23		
5	1.5 Gm	178	6.72 $\pm$ 0.33		
6	3.0 Gm			170	5.40 $\pm$ 0.26
7	5.0 Gm			172	8.11 $\pm$ 0.32

\* The riboflavin and pyridoxine series were run earlier than the others, using a somewhat heavier culture suspension this accounts for the greater number of organisms ingested

In every series of rats, deficiency of any one vitamin sufficient to retard growth also caused a reduction in phagocytic activity of the white blood cells. This reduction was most marked in the riboflavin and pyridoxine series in which the rats had been kept on the deficient diets for seven weeks before being tested. In thiamin deficiency of four weeks duration, there was a marked reduction in ingestion rate.

Group differences of 2 or more in the number of bacteria ingested per cell are of

unquestionably mathematical significance in the rat data here set forth. With hot-room groups 1 and 4 of the pyridoxine series, for instance, the difference is 7.67  $\pm$  0.55, the difference being fourteen times its own probable error and with only an infinitesimal likelihood of ever occurring by chance along. Even the difference of 4.11  $\pm$  0.34 (cold-room groups 1 and 3 of the thiamin series) is twelve times its own probable error.

The testing of each series of rats was done in the course of a single half day, using one dilution of the bacterial culture for all tubes. It is conceivable that many of the organisms might have died during the course of the two hours or so elapsing between the running of the first and last groups of the series, thus accounting for the rising ingestion rate. However, no significant difference was found when the culture suspension was tested on normal bloods after standing for intervals up to five hours after the dilution was made, nor did the use of a heat-killed culture alter the rate of ingestion. Furthermore, the order of testing was always to finish the groups of one room and then go on to those of the series in the other room. It is thus not possible to account for any of the observed differences on the basis of change in the culture. The samples of heparinized blood stood about fifteen minutes on the average before being run, but even five hours of standing at room or water-bath temperature had no effect on white cell activity.

We have made no use of continuous observations of phagocytic activity of single cells, since different neutrophils of the same animal may show rather marked variations in activity. The statistical approach seemed more appropriate and has been used throughout. In registering ingestion counts, any cell was considered full when it contained 30 or more bacteria. Beyond this point, accurate counting became impossible because of the crowding.

In the entire absence of any vitamin, phagocytic counts often rose from their usual low level when the condition of the deficient animal became critical. It is to be noted also that our highest ingestion-counts were always obtained in animals receiving the diet richest in the vitamin concerned. Studies are now in progress to see whether this relationship would continue with still higher concentrations of vitamin in the diet.

In addition to the studies shown in table 1, preliminary tests have indicated that lack of vitamins A and D (combined) produces a similar reduction in phagocytic activity. Inositol and p-aminobenzoic acid have so far been found to be without effect.

*Effects of vitamin C deficiency on phagocytosis in guinea pig blood.* Four groups (4 to the group) of young guinea pigs were placed on a basal diet consisting of wheat bran (45 per cent), rolled oats (25 per cent), and dried skimmed milk (30 per cent). Three drops of haliver oil were given weekly to supply vitamins A and D. One group in the hot room and one in the cold were given plenty of leafy vegetables in addition to the basal diet, while the second group in each room got no vitamin C. After three weeks, the weight of those getting no vitamin C showed no gain, as contrasted of an average gain of about 60 grams per pig in the control groups. Phagocytic tests at the end of the 3-week period gave the ingestion findings, as shown in table 2, using the same technic as in the rat studies.

A second series of guinea pigs were kept in the rooms on the basal diet for four weeks. Graded amounts of ascorbic acid were given daily by pipet, these amounts being 0.5 mg, 1.5 mg, and 3.0 mg per day per pig. All those in the cold room receiving no ascorbic acid died near the end of the fourth week before they had been tested for phagocytic activity. Those of the corresponding hot-room group died during the fifth week. Tests on those remaining alive at the end of the fourth week gave the results as shown in table 3.

The guinea pig bloods were highly unsatisfactory to work with because of troublesome clumping and fragmentation of the phagocytic cells. Even with all due reservations as to the accuracy of the counts, however, there is no doubt of a marked reduction in phagocytic activity in severe vitamin C deficiency.

TABLE 2

	Number of bacteria ingested per cell
Cold room full diet	18.30 $\pm$ 0.23
no vitamin C	7.30 $\pm$ 0.30
Hot room full diet	16.12 $\pm$ 0.44
no vitamin C	8.20 $\pm$ 0.28

TABLE 3

Ascorbic acid	Bacteria per cell at 68 F	Bacteria per cell at 90-91 F and 60-70% rel. hum.
mg / pig/day		
0.0		5.02 $\pm$ 0.38
0.5	7.42 $\pm$ 0.56	15.53 $\pm$ 0.69
1.5	11.90 $\pm$ 0.38	17.86 $\pm$ 0.79
3.0	12.02 $\pm$ 0.59	19.27 $\pm$ 0.81

*Protein deficiency studies in rats* Increasing emphasis is now being placed on the role of body proteins in resistance to infection and on the maintenance of acquired immunity. Cannon's excellent discussion of the subject pictures the loss of protein-attached immune bodies as tissue reserves of protein are depleted from any cause (blood loss, protein starvation, and the malnutrition accompanying vitamin deficiency, debilitating disease or old age). No mention has been made, however, of the part reduced phagocytosis might play in such loss of resistance. Hence, we decided to study this phase of the subject in conjunction with our work on vitamin deficiency and differences in protein requirement in heat and cold.

Using the phagocytic technic and basal diet described in the preceding pages, we adjusted the protein- and sugar content of the basal diet to give 6, 12, 18, 24, and 36 per cent protein and corresponding reductions in sugar. All vitamins were kept at optimal levels, with the needed increases in thiamin and choline in the hot room. Weanling white rats (males) were placed on these diets in the hot and cold rooms in groups of 4. After five and one-half weeks on the diets, the rats were bled and phagocytic tests run as described. The data obtained are as shown in table 4.

Here we see best phagocytosis and best growth taking place at 18 per cent dietary protein in the cold-room rats, with slight growth impairment and marked reduction in phagocytic activity as protein intake is increased above this level. In the hot room, on the other hand, both growth and phagocytic activity continue to improve with rising protein intake, even up to the 36 per cent level. While the differences in phagocytosis between contiguous groups of rats are not mathematically significant, those between the high and low groups of each room are highly so. The difference between groups 1 and 3 of the cold room ( $2.98 \pm 0.37$ ) is eight times its own probable error and would occur by chance only once in 14,700,000 times. Similarly, the difference of  $3.56 \pm 0.39$  for hot-room groups 1 and 5 is nine times its own probable error. Just why phagocytic activity should decline with the higher protein intakes in the cold must be left for future explanation.

TABLE 4

	Protein in diet	Bacteria per cell	Aver wt after 5½ weeks
	%		gm
At 68 F	6	$3.54 \pm 0.22$	70
	12	$3.76 \pm 0.24$	156
	18	$6.52 \pm 0.30$	206
	24	$4.66 \pm 0.21$	205
	36	$3.28 \pm 0.21$	201
At 90-91 F and 60-70% relative humidity	6	$3.76 \pm 0.23$	51
	12	$4.78 \pm 0.24$	100
	18	$5.62 \pm 0.23$	157
	24	$5.39 \pm 0.26$	195
	36	$7.32 \pm 0.32$	203

From these observations it seems evident that protein intake is fully as important as proper vitamin supply in maintaining optimal phagocytic activity. The casein used here as the total protein supply is poor in cystine, but even when 0.2 per cent cystine is added to all diets there still is evidence of a higher protein requirement in the heat than in the cold.

*Time lag in phagocytic response to changes in nutritive state.* Preliminary observations had indicated that full vitamin-deficiency effects on phagocytosis would be in evidence after four weeks on the deficient diets. It seemed desirable, however, to have more definite information on the time relationships involved.

Weanling white rats (Sprague-Dawley males) were placed in tropical warmth (90 to 91 F and 60 to 70 per cent relative humidity) and kept on synthetic diets for eight months before being used for the study. The rats in one group received the optimal diet for tropical warmth described previously, while those in the other group received a diet moderately deficient in protein and all the B vitamins (table 5).

While this low-vitamin rat diet would appear to be only mildly deficient, it was about as low as would be tolerated by 8 month old rats. Further reduction of



thiamine from 12 mg per kilogram down to 10 mg per kilogram resulted in typical severe deficiency symptoms and death within four to five weeks. Rats of this age, kept since weanlings in the heat on the optimal hot-room diet, also develop fatal thiamine deficiency in about the same time if the dietary thiamine is reduced to 10 mg per kilogram.

Using the technic described above, we measured the phagocytic activity of the blood polymorphonuclear neutrophils and then placed the rats with deficiencies

TABLE 5 — *Amounts of B-vitamins and Casein per Kilo of Diet*

	Optimal diet for tropical warmth	Deficient diet
Thiamine hydrochloride	2.5 mg	1.2 mg
Riboflavin	4.0 mg	1.5 mg
Pyridoxine	4.0 mg	1.5 mg
Calcium pantothenate	6.0 mg	1.5 mg
Nicotinic acid	25.0 mg	10.0 mg
Choline chloride	5.0 Gm	1.0 Gm
Inositol	1.0 Gm	0.4 Gm
p-Aminobenzoic acid	0.3 Gm	0.1 Gm
Casein vitamin free	180 Gm	120 Gm

TABLE 6 — *Weekly Changes in Phagocytic Activity*

	Control rats on full diet	Time on new diet	Rats changed from full to deficient diet		Rats changed from deficient to full diet	
	Mean number of bacteria per cell		Mean number of bacteria per cell	% of normal	Mean number of bacteria per cell	% of normal
		weeks				
	6.12 ± 0.36	1	6.59 ± 0.33	108	2.65 ± 0.18	43
	6.17 ± 0.37	2	5.04 ± 0.27	82	4.10 ± 0.31	66
	5.37 ± 0.33	3	2.51 ± 0.20	47	5.51 ± 0.28	103
	7.57 ± 0.37	4	2.87 ± 0.19	38	8.32 ± 0.29	110
Wt changes						
First week			-10 gms		+17 gms.	
4 weeks	+16 gms		-68 gms		+61 gms.	
Initial average wt	340 gms		346 gms		169 gms	

on the optimal diet while changing some of the normal rats to the deficient diet. Estimates of phagocytic activity and weight change were made weekly thereafter for each group. Four rats in each group were bled from the heart and discarded from the study at the end of each week so that the study would not be complicated by any possible effect on phagocytosis from repeated bleedings.

In table 6 are set forth the changes in phagocytic activity and body weight which took place from week to week. Even though the dietary shifts in each case were promptly reflected in body weight changes within the first week, no corresponding

alterations were found in activity of the blood phagocytes. By the end of the second week a moderate change was observed in phagocytic function; this became more marked during the third week and was complete by the end of the fourth week (fig 1). After four weeks, the rats that formerly had deficiencies exhibited normal phagocytic activity, while those previously normal were now at the low level of full deficiency.

With bacterial counts made in 40 phagocytes from each rat (making 160 cells for each mean value recorded in the table), differences in one day's readings greater than 15 became statistically significant. Naturally the readings of one week can be compared with those of another week only by reference to each week's normal values obtained on the control rats. The bacterial suspension used in the third week's test was slightly too dilute, while that of the fourth week was distinctly

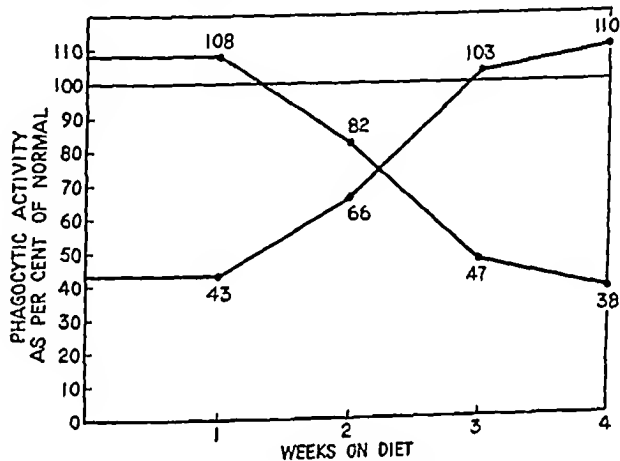


FIG. 1. Phagocytosis in rat malnutrition and recovery

heavy. This necessitated the calculation of comparative changes on a percentage basis, using the normal controls as 100 per cent in every case. Differences of  $\pm 10$  per cent are of no definite significance here.

One review journal has recently<sup>3</sup> criticised our use of only 40 cells per rat (four rats to each group) as the basis for calculating mean ingestion rates, referring to the custom of counting ingestion from 200 to 500 cells in human phagocytic studies. To test the stability of our ingestion values, we selected two representative groups from our previous report, Groups 1 and 3 of the riboflavin series in the cold room. In addition to the mean values calculated on counts from 160 cells per group (40 per rat), we recalculated the data on the basis of including only the first 20 (10, 5, and 2 phagocytes seen on each slide). In table 7 are set forth the results of this recalculation, and it is clearly indicated that the observed differences in mean ingestion counts maintain their statistical significance when as few as only the first 10 cells per rat are included in the calculation. It may thus safely be accepted as

true that counts made on 40 phagocytes per animal, with four animals to each group, allow an ample margin of stability. In human case studies, 100-cell counts would provide sufficient stability if the technical details of the method were carefully standardized.

TABLE 7

No. cells counted		Group 1		Group 3		Group diff in means	Size of diff in means needed for significance (P.E. X 4)
Per rat	Per group	Mean number bacteria ingested per cell	Standard deviation of mean	Mean number bacteria ingested per cell	Standard deviation of mean		
40	160	5.76 $\pm$ 0.27	5.12 $\pm$ 0.19	9.37 $\pm$ 0.28	5.28 $\pm$ 0.20	3.61 $\pm$ 0.39	1.56
20	80	5.23 $\pm$ 0.38	5.03 $\pm$ 0.27	8.88 $\pm$ 0.37	4.97 $\pm$ 0.26	3.65 $\pm$ 0.53	1.12
10	40	4.55 $\pm$ 0.52	4.92 $\pm$ 0.37	8.10 $\pm$ 0.51	4.77 $\pm$ 0.36	3.55 $\pm$ 0.73	2.92
5	20	6.20 $\pm$ 0.86	5.72 $\pm$ 0.61	7.90 $\pm$ 0.85	5.63 $\pm$ 0.60	1.70 $\pm$ 1.21	4.84
2	8	6.25 $\pm$ 1.46	6.12 $\pm$ 1.03	6.75 $\pm$ 1.14	4.79 $\pm$ 0.81	0.50 $\pm$ 1.85	7.40

### DISCUSSION

From the results here presented, it would seem that the ability of bone marrow to produce active phagocytes is dependent on the same nutritional requirements needed for optimal body growth. Deficiency of any one of the B vitamins (except inositol or p-aminobenzoic acid) or of protein sufficient to retard body growth also interferes with the marrow production of normally active phagocytes.

This effect of nutritional deficiency seems to be exerted only upon phagocytes during their early immature period in the marrow tissue, for nutritional correction—which at once restores normal growth—fails to bring back normal phagocytic activity to circulating granulocytes except after a lag of between two and three weeks. It therefore seems justifiable to conclude that granulocytes already in the circulating blood are not influenced by changes in nutritional status, that they are susceptible only during their early formative period. This means that improved phagocytosis cannot be expected until two to three weeks after nutritional faults have been corrected.

Fewer, as well as less active, phagocytes are produced in deficiency states, the total leukocyte counts in rats dropping from a normal level of around 10,000 per cubic millimeter down to about 4,000, without any marked change in the differential count.

Preliminary observations have shown phagocytosis to be 4 to 5 times as active in some human subjects during the summer months than through the winter season. Whether this winter decline in activity is related in a causative way to the greater susceptibility to colds and other respiratory infections, forms interesting grounds for speculation.

### CONCLUSIONS

Deficiency of any of the B-vitamins (except inositol or p-aminobenzoic acid), vitamin C, or of protein, leads to a reduction in phagocytic activity of the blood.

granulocytes in experimental animals. This effect of a faulty diet, or of a restoration to normal after a period of malnutrition, alters the phagocytic activity of circulating granulocytes only after a lag of two to three weeks, thus leading to the conclusion that these cells are susceptible to nutritional faults only during their early formative period in the marrow tissue.

## REFERENCES

- <sup>1</sup> COTTINGHAM, ESTHER, AND MILLS, C. A. Influence of environmental temperature and vitamin deficiency upon phagocytic functions. *J. Immunol.* 47: 493-502, 1943.
- <sup>2</sup> CANNON, PAUL R. Antibodies and the protein reserves. *J. Immunol.* 44: 107-114, 1942.
- <sup>3</sup> EDITORIAL. Effects of nutrition on phagocytosis. *Nutrition Rev.* 2: 232-4, 1944.

# SOME OBSERVATIONS ON THE EFFECT OF FOLIC ACID ANTAGONISTS ON ACUTE LEUKEMIA AND OTHER FORMS OF INCURABLE CANCER

By SIDNEY FARBER, M D

THE PRODUCTION of temporary remissions in the course of acute leukemia in children by the administration of the compound, 4-aminopteroylglutamic acid (aminopterin)<sup>1, 2</sup>—a biologic antagonist to folic acid\*—has raised a number of theoretic and practical questions. Confirmation of this finding has been reported from several sources<sup>3</sup>, temporary remissions equally impressive have been obtained in adults with acute leukemia by Dameshek.<sup>4</sup>

It is the purpose of this paper to summarize briefly the status of our observations† on the action of folic acid antagonists on acute leukemia and other incurable forms of cancer for the interest of those now working with these agents, to state the nature of some of the problems which have arisen, and to indicate some directions of further research.

The demonstration by Lewisohn and his colleagues<sup>5</sup> of the occurrence of complete regression in about one-third of single spontaneous breast cancers in three different strains of mice treated with fermentation L casei factor, later shown to be pteroyltriglutamic acid (Hutchings et al.<sup>6</sup>) and the subsequent synthesis of this compound by SubbaRow and his co-workers<sup>7</sup> led to our study of the effect of pteroyltriglutamic acid on incurable cancer in man. Among the patients so treated were 11 children with acute leukemia. The occurrence of what we called an acceleration phenomenon in the viscera and bone marrow of these patients and an experience with folic acid deficiency experimentally produced in the rat suggested that it would be worth while to ascertain if this acceleration phenomenon might be employed to advantage in the treatment of acute leukemia in children, either by the use of radiation or nitrogen mustard therapy after pretreatment with folic acid or conjugates of folic acid, or by the immediate use of folic acid inhibitors or

From The Children's Medical Center, Boston, Mass. and The Department of Pathology, Harvard Medical School.

Presented in part at the Congress of the International Society of Hematology, Buffalo, New York, August 25, 1948.

This study has been supported in part by Cancer Grant #250-390 of the National Cancer Institute, United States Public Health Service and in part by The Children's Cancer Research Foundation, Boston.

This paper is dedicated to Dr. George R. Minot. It was my privilege when a student to hear his lectures on diseases of the blood. In these he united in masterful fashion the fields of pathology, physiology, and clinical medicine to establish a logical approach to the nature of disease and so in therapy. His announcement, when I was a fourth year student, of the liver treatment of pernicious anemia fired the imagination of all who heard him to a consideration of the role of nutrition in other incurable diseases of unknown etiology. S. F.

\* By antagonist to folic acid is meant a substance which possesses the property of inhibiting the growth of *Streptococcus Faecalis R.* or *L. casei* in the presence of marginal levels of folic acid. Reversal of inhibition occurs when the concentration of folic acid in the culture medium is elevated.

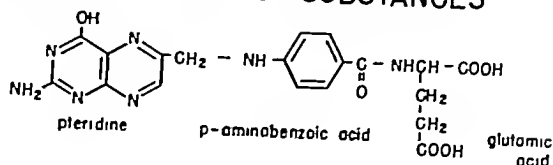
† Our studies represent the accomplishment of a group of clinicians and laboratory workers who have joined forces to make possible rapid progress along the lines indicated in this paper. Detailed reports of clinical, experimental, toxicologic and pathologic studies are being prepared for publication.

## NUTRITIONALLY ACTIVE SUBSTANCES

PTEROYL  
GLUTAMIC  
ACID (*PGA*,  
*Folic Acid*)

PTEROYL  
DIGLUTAMIC  
ACID (*PG<sub>2</sub>*,  
*Diopterin*)

PTEROYL  
TRIGLUTAMIC  
ACID (*PG<sub>3</sub>*,  
*Teropterin*)

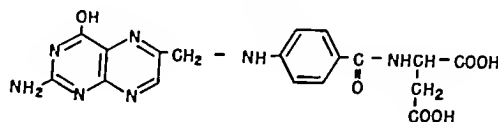


pteridine - p-aminobenzoic acid - two glutamic acids joined by peptide links

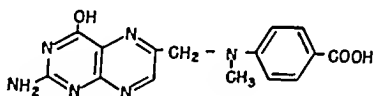
pteridine - p-aminobenzoic acid - three glutamic acids joined by peptide links

## BIOLOGICAL ANTAGONISTS

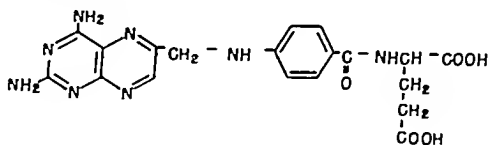
PTEROYL  
ASPARTIC  
ACID  
(*An-Fol A or R*)



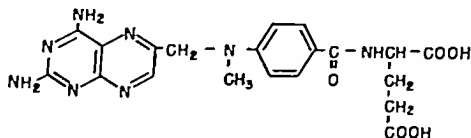
METHYL  
PTEROYL  
ACID  
(*Mel-Fol B*)



4-AMINO  
PTEROYL  
GLUTAMIC  
ACID  
(*Aminopterin*)



4-AMINO  
METHYL  
PTEROYL  
GLUTAMIC  
ACID  
(*A-Methopterin*)



4-AMINO  
PTEROYL  
ASPARTIC  
ACID  
(*Amino-An-Fol*)

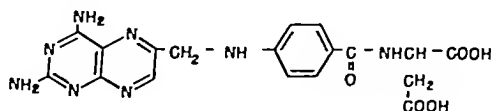


FIG 1

antagonists \* The first folic acid antagonists—pteroylaspatic acid and methyl pterotic acid—were effective enough not only to give the needed encouragement for further research in this direction, but also to prolong the lives of a few children with acute leukemia until more powerful antagonists of folic acid were made available The first impressive remissions in the course of acute leukemia were produced by the use of aminopterin beginning in November of 1947 These were characterized by a return almost to a normal state in some and to a state almost indistinguishable from normal in others in a group of 10 of 16 children with acute leukemia The toxicity of aminopterin emphasized the need for less toxic compounds which it was hoped might be even more effective in their carcinolytic action<sup>1,2</sup>

### *Compounds Related to Aminopterin*

Observations have been made on children with acute leukemia and on patients with a variety of other forms of incurable cancer treated by two compounds closely related to aminopterin Both of these were supplied by the late Dr Y SubbaRow These are 4-aminopteroylglutamic acid (amethopterin) and 4-aminoaspatic acid (amino-an-fol) \* A complete account of these studies will be presented elsewhere In general it may be stated that while amethopterin and amino an fol are less toxic than aminopterin, exactly the same toxic changes may be produced when appropriate doses are employed This holds true for laboratory animals and for man Remissions in the course of acute leukemia in children equal to those produced by aminopterin may be brought about by the use of amethopterin or amino an fol The effective dose when remissions are obtained in children with acute leukemia lies between 3 to 5 mg a day for amethopterin, and between 25–30 mg a day for amino an-fol, depending upon age, weight, size, and physical condition of the patient These figures may be compared with a range of 0.5 mg to 1.0 mg a day of aminopterin Because there is some differential in the dose required to produce toxic changes, as compared to the effective dose, it has been possible to shift from one drug to another when early signs of toxicity have become apparent

### *Pattern of Therapy*

It is impossible to present at this time a pattern of therapy as definite as that governing the use of digitalis, for example, or insulin Daily white count and physical examination are the best guides to the treatment to be given that day Too rapid a drop in the white count, diarrhea of unknown origin, the presence of stomatitis, a sore tongue, or ulceration of the mucous membranes of the mouth,

\* Acknowledgement is made to the late Dr Y SubbaRow and his colleagues in the Research Division of the Lederle Laboratories (American Cyanamid Company) and their associates of the Calco Chemical Division, who are responsible for the chemical research that made possible these studies on children A particular word of gratitude is expressed not only for the invaluable chemical contributions of Dr SubbaRow but also for his decision to pursue so effectively by further chemical research the leads which were obtained from these studies on children with acute leukemia The present plan of study concerning the action of folic acid antagonists is following along the lines decided with Dr SubbaRow in the spring of 1947 It consists essentially of the study of the action on laboratory animals and on patients with various forms of incurable cancer of related compounds in an attempt to find one which is more effective and less toxic than any we have previously employed

should serve as reasons for cessation of therapy until the exact cause for these disturbances has been determined. In periods of remissions treatment continues as before, although slightly smaller doses may be administered. In some instances when patients are doing well, intramuscular injection of the compound employed has been given on every other day. Aminopterin apparently is effective also when given by mouth.

### *Toxicity*

Our initial report carried a warning concerning the toxic nature of aminopterin. Stomatitis, ulceration of the mucous membrane of the mouth, smooth tongue, pharyngitis, and atrophic changes in the intestinal epithelium of the type produced by folic acid deficiency in the rat and in the monkey, diarrhea, gastro-intestinal hemorrhage, particularly when there is diffuse leukemic infiltration of the bowel, and depletion of the bone marrow leading to aplasia are the most important changes. Despite efforts to prevent or to overcome quickly the toxic manifestations by the use of liver extract, various vitamin B preparations and folic acid itself in doses up to 200 mg. a day for several days, the most effective treatment appears to be suspension of administration of aminopterin for four to seven days at the first sign of stomatitis or diarrhea of unexplained origin.

The occurrence of hypersegmented polymorphonuclear leukocytes and the presence in the bone marrow of megaloblasts have been observed as important evidences of the effect of the antagonist. It is impossible to state at this time with certainty whether all of the changes produced in acute leukemia by antagonists to folic acids are manifestations simply of a folic acid deficiency. It does appear that the alterations are at the same time more profound and more subtle than those produced by folic acid deficiency alone and that interference with biochemical systems more important than simple competitive substitution of the antagonist for folic acid within cells must obtain. Evidence bearing on this point is being collected.

### *Hemorrhage*

Hemorrhage into the gastro-intestinal tract, the skin, and the genito urinary tract and the cranial vault, either massive or oozing in character, has always been one of the most serious complications of acute leukemia and one of the important causes of death. Studies now being conducted by our group following the work of Allen and Jacobsen<sup>9</sup> show that in many children with acute leukemia the level of heparin-like substances in the blood is definitely higher than the normal. While bleeding occurs usually when the level of blood platelets is low, thrombocytopenia may be present without any evidence of bleeding for many months. The longer survival of patients with acute leukemia made possible by folic acid antagonist therapy has brought the problem of hemorrhage into great prominence. The combination of leukemic infiltration of the intestinal tract and toxic effects produced by aminopterin, amethopterin, and amino-an-fol makes for the ready occurrence of gastro-intestinal hemorrhage. Although the exact explanation is not clear it appears certain that hemorrhage occurs more readily if the bone marrow is markedly depressed by the compound employed. The effect may be similar to that of



duced in aplastic anemia where gastro-intestinal hemorrhage is a common and serious occurrence. If toxic levels of the folic acid antagonist are employed long enough, the bone marrow may be depressed enough to accentuate the hemorrhagic tendency in leukemia, or to act as the sole cause of the hemorrhage.

### *Nature of Leukemia*

Observations on a girl (M. D.), 8 $\frac{1}{2}$  years old at the time of her death, and similar experiences with other children have raised a question concerning present conceptions of leukemia. This child lived for twenty-two months after the onset of acute leukemia. Treatment with pteroylaspartic and methylpterotic acid was followed by repeated temporary periods of improvement. She died following uncontrollable oozing from the mucous membranes. Postmortem examination revealed leukemic cells so few in number, in scattered areas throughout the body that the diagnosis of acute leukemia would have been made with hesitation on the basis of that evidence alone. It seems probable that hemorrhage in acute leukemia may be produced by a number of different factors apart from the effect of leukemic infiltrates on the bone marrow and viscera and the thrombocytopenia. The hypothesis seems warranted, that a serious disturbance in the hematopoietic system, or a series of deficiencies in the body responsible for oozing or for massive hemorrhage might still be present in the patient with acute leukemia if every leukemic cell in the body could be destroyed. Acute leukemia, therefore, may be a form of cancer complicated by specific deficiency states—a suggestion that has definite implications for further research.

### *Types of Leukemia*

In the majority of the children with acute leukemia treated it was impossible to diagnose with certainty the exact morphologic type of leukemia because of the primitive nature of the blasts. It would seem logical, and certainly highly desirable to replace or to supplement the morphologic classification of leukemia by one based upon response to specific stimuli, such as the folic acid antagonists. Study of those patients with acute leukemia who failed to respond to these compounds might yield data of value concerning the nature of the disease. A worthy goal is the characterization of the various types of acute leukemia in terms of precise intracellular biochemical deficiencies or alterations.

### RESULTS

In a group of approximately 60 children with acute leukemia treated for three weeks or longer with either aminopterin, amethopterin, or amino-an fol, some what more than 50 per cent showed improvement clinically, hematologically of important degree attributable to the action of these compounds. Detailed tabulations of our entire experience with thorough documentation will appear separately. Two of the five children whose case histories were presented in our initial report are still alive (December 21, 1948). Case 1 of that report, a boy of 8, has a history of acute leukemia beginning in February 1947. He was treated first with methylpterotic acid and pteroylaspartic acid. Aminopterin was not given until December

16, 1947 Since then that, or one of the other more powerful folic acid antagonists have been employed Leukemia is still present and there have been many complications, but he is still alive twenty-three months after the onset of his disease A second child mentioned in the earlier report, Case 5, has had acute leukemia since August, 1947 He is one of twins and despite his leukemia and almost constant folic acid therapy, he is as tall and as well nourished as his brother His leukemia, which is still recognizable by studies of bone marrow and peripheral blood, is still under control sixteen months after onset

The widespread use today of aminopterin in the treatment of acute leukemia has raised for discussion a basis of comparison of results Any evaluation of treatment of patients with incurable cancer must rest upon a solid foundation of knowledge concerning the life history and biologic behavior of tumors Acute leukemia, which runs an invariably fatal course, varying from a few weeks usually to six months after onset of symptoms, lends itself readily to comparative studies Rarely the course may last as long as twelve months, and isolated instances of longer survivals have been observed The end point of time itself, therefore, should serve as a reliable criterion of the value of any form of therapy

Spontaneous remissions, either complete or partial, occurred in 10 per cent of 300 children with acute leukemia observed by Dr Louis K Diamond,<sup>10</sup> at the Boston Children's Hospital These averaged slightly less than ten weeks in duration In two instances a second remission was observed In almost 75 per cent of these children in whom spontaneous remission was noted, there was a history of infection of important degree immediately preceding the remission The recent production of remission in acute leukemia by the use of massive blood transfusion makes necessary the evaluation of this factor too, in patients treated with folic acid antagonists Analysis of our experience permits the statement that the remissions we have described are dependent neither upon infection nor transfusions of blood

It is obvious that no two children with acute leukemia present strictly comparable problems Infiltration of the leukemic processes is generalized but there are great variations in the degree and site of involvement In one, a large subdural accumulation of tumor may alter intracranial pressures to an important degree, in another, the leukemic infiltration in the heart may be responsible for unexpected death Other variables are the amount of replacement of the bone marrow by leukemic cells, the factors responsible for bleeding, and the occurrence of secondary infections It should not be surprising, therefore, if one research group reports five consecutive remissions (personal communication from Dr George Guest, Cincinnati Children's Hospital), or that another group observes a fatal outcome within two weeks after onset of therapy in ten consecutive patients before one remission is observed The arbitrary limit of three weeks after onset of therapy has been chosen for a basis of comparison During this period those patients most severely involved will have died or the folic acid antagonists employed will have had an opportunity to effect the tumor infiltrations in the viscera and the bone marrow

It should be emphasized that all available resources of medicine have been utilized in an attempt to prolong the lives of our patients with acute leukemia Transfusions, radiation therapy, antibiotics and specialized dietary measures have all been

employed when indicated. It has been possible, however, to study a sufficient number of patients for long enough periods of time with folic acid therapy alone to permit the accumulation of sufficient data upon which reliable conclusions could be based. *It should be expected, therefore, that considerable variation in the results of different investigators will be reported until a sufficiently large experience has been obtained, or until a long enough period has elapsed to permit the use of the period of survival alone as the simplest criterion of therapeutic effect.*

The effect of these folic acid antagonists, despite some theoretic considerations which entered into the formulation of early working hypotheses, is not limited to acute leukemia. We have reported<sup>11</sup> temporary, definite but inconstant carcinolytic action on patients with apparently unrelated forms of incurable cancer, such as neuroblastoma, and pulmonary metastases from cancer of the bladder, as well as more closely related tumors such as lymphosarcoma and Hodgkin's disease.

The range of carcinolytic action on various types of incurable cancer in man is now being evaluated. The combined action of the folic acid antagonists when employed with other agents used in the treatment of cancer, such as the sex hormones and radiation therapy is under study.

The toxic nature of the compounds employed in these studies and the inconstant and temporary nature of beneficial effects make clear that the value of these compounds is still limited to research. The finding of equally or more effective and less toxic compounds, and an understanding of the reasons for failure in those patients who do not respond are goals which must be reached before more widespread application of the results of these studies is possible.

#### SUMMARY

A general discussion is presented of the present status of folic acid antagonist therapy in acute leukemia in children and in other forms of incurable cancer. Conclusions reached in our initial report have been supported by a far greater experience. Temporary remissions in acute leukemia as marked as those caused by aminopterin have been produced by the use of two compounds closely related chemically to aminopterin—amethopterin and amino-an-fol, both of which, however, are also toxic compounds. Despite the increasing number of patients in whom temporary remissions have been produced, with survival in some far beyond the usual course of the disease, no evidence has been presented which would justify the use of the word "cure" of acute leukemia. A carcinolytic action on related and on certain unrelated forms of incurable cancer has been observed. Further research for less toxic related compounds with even greater effectiveness is not only justified by these studies but is imperative. The value of this direction of research in cancer has been established.

Two of the most pressing problems demanding solution are concerned with the nature, the prevention, and the treatment of toxic changes, including hemorrhage, produced by these folic acid antagonists and the causes, prevention and mechanism of hemorrhage in acute leukemia. The use of the folic acid antagonists in the treatment of incurable cancer including leukemia must remain in the realm of research until answers to these questions are found.

## REFERENCES

- <sup>1</sup> FARBER S Temporary remissions in acute leukemia in children produced by folic acid and antagonists Proc Div Laboratories and Research Children's Medical Center Boston Mass 1 1-5, 1948
- <sup>2</sup> —, DIAMOND L K, MERCER, R D, SYLVESTER, R F JR AND WOLFF, J A Temporary remissions in acute leukemia in children produced by folic acid antagonist, 4 aminopteroyl glutamic acid (aminopterin) New England J Med 381 787-93 1948
- <sup>3</sup> Discussion to paper by FARBER SIDNEY at First International Hematological Congress, Buffalo, New York August 25 1948 by Drs William Dameshek, Robert Heinle, Byron Hall, Werner Jacobson and George Guest
- <sup>4</sup> DAMESHEK WILLIAM (Editorial) The use of folic acid antagonists in acute leukemia Blood 3 1057, 1948
- <sup>5</sup> (a) LEUCHTENBERGER C., LEWISOHN R, LASZLO D, AND LEUCHTENBERGER R Proc Soc. Exper Biol & Med 55 204 1944
- (b) LEUCHTENBERGER R, LEUCHTENBERGER C, LASZLO D, AND LEWISOHN R Science 101 46 1945
- (c) LEWISOHN, R, LEUCHTENBERGER, C, LEUCHTENBERGER R AND KERESZTESY J Science 104 436 1946
- <sup>6</sup> HUTCHINGS, B L., STOKSTAD, E L R, BOHANNON N AND SLOANE N H Science 99 371 1944
- <sup>7</sup> ANGIER, R B et al Science 103 667 1946
- <sup>8</sup> (a) HUTCHINGS, B L et al Pteroylaspartic acid antagonist for pteroylglutamic acid J Biol Chem 170 323-328, 1947
- (b) SEEGER, D R, SMITH, J M JR AND HULTQUIST M E Antagonist for pteroylglutamic acid J Am Chem Soc 69 2567 1947
- (c) SMITH J M JR, COSULICH D B, HULTQUIST M E AND SEEGER, D R Chemistry of certain pteroylglutamic acid antagonists Tr New York Acad Sc Special Number Series II, 10 82, 1948
- <sup>9</sup> ALLEN, J G, AND JACOBSON, L O Hyperheparinemia Cause of the hemorrhagic syndrome associated with total body exposure to ionizing radiation Science 105 #27 28 April 11 1947
- <sup>10</sup> DIAMOND LOUIS K Personal communication
- <sup>11</sup> FARBER SIDNEY Annual Meeting, American Cancer Society, November 7 1948, New York City In press

# THE USE OF FOLIC ACID ANTAGONISTS IN THE TREATMENT OF ACUTE AND SUBACUTE LEUKEMIA

## A PRELIMINARY STATEMENT

By WILLIAM DAMESHEK, M D

A RECENT meeting of the New York Society of Hematology held on November 30, 1948, was devoted to a symposium on the treatment of leukemia with aminopterin. It was obvious that definite remissions induced by the drug and not of spontaneous nature were being obtained, although the results of therapy in the hands of various workers differed considerably.

In this issue Farber presents a summary of his results with various folic acid antagonists. Since Farber's observations deal almost wholly with children and our own work has been almost wholly with adults, it was thought that a preliminary statement of our own results with these drugs as reported at the above meeting might be of interest.

Thirty five cases of acute and subacute leukemia including 4 children and thirty one adults, are or have been under treatment with one or more of the folic acid antagonists since mid-April 1948.\*

The folic acid antagonists used were 4 amino, pteroyl glutamic acid—(aminopterin), 4 amino, N<sup>10</sup> methyl pteroyl glutamic acid (a-methopterin), 4 amino, pteroyl aspartic acid—(amino-an-fol), and 4 amino, 9 methyl pteroyl glutamic acid—(a-ninopterin).

These chemicals, dissolved in sterile normal saline, were injected intramuscularly daily until a toxic or pronounced hematologic reaction occurred, following which the drug was discontinued. The drug was then resumed in a maintenance dose when the toxic reaction had subsided. Aminopterin was given in a dosage of 1-4 mg daily, a-methopterin, 2-5 mg daily, amino-an-fol 25-75 mg daily, and a-ninopterin 5-15 mg daily. Maintenance therapy was given either daily or every other day and either by intramuscular or oral route. Tablets of oral aminopterin were ordinarily used in 1 mg dosage.

Of the 35 cases of acute and subacute leukemia, 1 has been under treatment for less than four weeks, leaving 34 cases for analysis. Of these, 8 died within one to five days after therapy was instituted. Since death occurred so shortly after institution of drug therapy, these cases should probably be excluded from any statistical analysis of the therapeutic effects of the drug. If this is done, 26 cases of acute and

From the Ziskind Laboratories (Hematology Section) of the J. H. Pratt Diagnostic Hospital and the Department of Medicine Tufts College Medical School. Aided by Grants from the American Cancer Society (Massachusetts Division), the Charlton Fund and the Lederle Laboratories. A detailed report of our findings is in preparation.

\* The detailed data obtained in these cases will be presented by Drs. William Dameshek, Milton Freedman and Lester Steinberg at a later date.

subacute leukemia are left for evaluation. Of these cases, 9 have had continued or intermittent remissions for at least two months and up to eight and one-half months (as of January 20, 1949). A remission is deemed present when the patient, (a) feels subjectively improved, (b) shows such objective clinical improvement as regression of lymphadenopathy and hepatosplenomegaly and loss of hemorrhagic tendency, (c) shows hematologic improvement as evidenced by improvement in the red cell count, return of leukocyte counts to relatively normal values, a definite increase in blood platelets, and an improvement in the marrow picture, and (d) shows continuous improvement for at least two months.

The remission rates are, therefore *Gross results* 34 cases (8 dying in one to five days), 9 remissions = 26 per cent. *Adjusted results* 26 cases (excluding those dying in one to five days), 9 remissions = 34 per cent.

In the early stages of the study, crude liver extract was used in the attempt to allay the toxic symptoms but this was soon discarded. Folic acid was also used in one case, but since it caused a quick relapse in the leukemic process, it was discarded after a single trial. Penicillin was given routinely in the presence of marked granulocytopenia and/or fever. Transfusions of blood were used to maintain the red cell count at levels of approximately 2.5 to 3.0 M.

The remissions occurring in the 26 cases cited above were further analyzed with respect to the proliferating cell type involved. This is often very difficult because of the primitiveness of the proliferative process. In the more recently studied cases, a battery of studies were carried out to determine this previously rather academic question. This included not only the use of the ordinary Romanowsky stains, but oxidase stains, supravital studies, histochemical staining methods including the use of sudan black and phase microscopy.

Best results with the folic acid antagonists were obtained in the lymphoblastic cases. None of the monocytic cases responded.

	Cases	Remissions
Lymphoblastic	10	5
Myeloblastic	9	3
Monocytic	3	0
Unclassified	4	1

The greater specificity of the folic acid antagonists for lymphoblastic proliferations is in line with a more or less marked specificity of certain of the chemotherapeutic agents now in use for certain cell types. Thus, nitrogen mustard appears to be most useful in reticulum cell proliferations including Hodgkin's disease, reticulum cell sarcoma, and reticulosis, urethane in granulocytic proliferation of the chronic myelocytic type and in plasmacytoma (multiple myeloma).

Following the development and then subsidence of the toxic reaction to the drug or following the appearance of a reasonably normal white blood cell count, or under both circumstances, a maintenance dose of the drug was given. In recent months, this was usually given by oral route, in a dosage of 1 mg daily or every other day. Oral aminopterin has proved to be equally as effective as the parenteral medication, causing as marked therapeutic and toxic effects, mg for mg, as when given parenterally.

Toxic reactions were the rule with aminopterin administration. These depended in great part on the dose used and were in the nature of ulcerative mucous membrane and tongue lesions, nausea, burning sensation in the upper abdomen and diarrhea, a form of vascular purpura and an apparent aggravation of the bleeding tendency.\* The marked reduction in leukocyte count and to lesser extent of the other blood elements might be considered as due to a preferential effect of the chemical on the bone marrow. Other folic acid antagonists, such as  $\alpha$ -methopterin and amino-an-fol were less toxic than aminopterin but in general of lesser therapeutic value.

The impression was obtained that in order to obtain a remission it was necessary to bring about definite so-called toxic manifestations. The margin of safety between a toxic reaction and death was at times very small.

It is the natural objective of the chemist to produce materials with relatively slight degrees of toxicity while maintaining at least a standard therapeutic effect. Recent observations indicate that such a possibility may be present in one of the methylated aminopterins (9 methyl, 4 amino PGA or  $\alpha$ -ninopterin). In at least one case given this material, therapeutic effects comparable with those of aminopterin were obtained with only minimal toxic effects.

#### SUMMARY

In summary, the folic acid antagonists have, in varying degrees, the capacity to induce remissions in about one third of the cases of acute and subacute leukemia, in adults as well as in children, and in both leukemic and leukopenic forms.

Clinical, hematologic and (to lesser extent) marrow remissions, are obtained most commonly in the lymphoblastic types, least often in the monocytic types.

It is possible that folic acid is required by the primitive white cell as a growth factor. The folic acid antagonists, which resemble folic acid so strikingly in chemical structure, may result in cell death by modifying various enzyme systems within the primitive cells.

Both clinical and hematologic observations indicate that the proliferative process is by no means cured with aminopterin treatment. Acute leukemia may be likened to wildfire which, although damped by aminopterin, continues to smolder. This smoldering may suddenly light up again into an active leukemic picture, unless continued maintenance therapy is given. Despite maintenance therapy, there finally comes a point in the leukemic process at which both the leukemia and increasing toxicity to drug make further progress impossible, and the patient dies.

Other growth factors or enzymes are probably of at least as great an importance as PGA in the metabolism of the primitive white cells and when these are discovered and their antagonists synthesized, the therapeutic results in acute leukemia may be of more consistent and durable nature. It should be realized further that chemotherapeutic methods against leukemia and the leukocytic proliferations in general (and, in fact, against all proliferative disease) are at least in their very

\* A toxic reaction may represent simply one or another aspect of the therapeutic response or the part of cells in various parts of the body to the folic acid antagonist. Tissues differ widely in their response to the chemical and leukemic tissue may be preferentially affected.

infancy The results thus far obtained in acute leukemia, although to large extent disappointing, indicate that well defined remissions can be secured in about a third of the cases For a disease such as acute leukemia, in which remissions previously were highly unusual and of sporadic nature, this indicates a well-defined therapeutic advance and a need for continued investigation along the same general lines

Differences in results obtained by various groups of workers are difficult to explain Several points may nevertheless be considered some of the workers have given inadequate dosage of drug or have failed to use maintenance therapy, some have given folic acid in conjunction with anti-folic acid therapy, in some cases, a crude folic acid antagonist was used, and it is possible that some cases were not observed as minutely as seems necessary An important factor, which can be determined only by the study of a large group of cases, is the natural variability of acute leukemia from case to case We have the impression that our best results are obtained in the relatively subacute cases The fulminating cases, with rapid onset of bleeding and a quick downhill course, are only slightly affected



# THE DISTRIBUTION CURVE OF ERYTHROCYTE FRAGILITY

## A DIFFERENT METHOD OF PRESENTATION OF FRAGILITY OF ERYTHROCYTES TO HYPOTONIC SALINE, WITH PRELIMINARY REMARKS ON THE FUNCTION OF RETICULOCYTES

By J H BOLTON M D

### INTRODUCTION

FOLLOWING Haden's<sup>10</sup> demonstration of the close correlation between spherocytosis and the fragility of erythrocytes to hypotonic saline, Dameshek and Miller<sup>5</sup> postulated that spherocytosis was a preliminary stage in the destruction of the red cell, i.e., partial hemolysis. They were able to show experimentally that the degree of spherocytosis was related to the concentration of hemolysins in the blood and that this was also correlated with fragility. They also described cases of acquired hemolytic anemias with spherocytosis and increased fragility, thus emphasising the danger of using spherocytosis and increased fragility as criteria for the diagnosis of familial acholuric jaundice. In addition, they demonstrated the presence of hemolysins in a number of cases of hemolytic anemia.<sup>6, 7, 8</sup>

The chain of reasoning is that hemolysins may lead to partial damage to the cell, this is followed by entry of fluid and loss of the biconcave disc form with approximation to a spherical shape. Fluid can enter a cell to a certain maximal degree, but if this is exceeded, the cell will rupture. Hence, if a cell is already partially spherical, it will rupture with a smaller entry of fluid than if it possesses the biconcave disc form. As the cell acts as an osmometer, the amount of fluid entering the cell will depend on the concentration of electrolytes on either side of the membrane, and this forms the basis of the fragility test with hypotonic saline.

### METHOD

As usually presented, the fragility curve is sigmoid in shape and alteration of form is difficult to assess. But this sigmoid shape is due to the fact that the curve is a composite one and at each decreasing concentration of saline the percentage hemolysis at any particular point is the sum of all the hemolysis which has occurred at higher concentrations of saline, plus the actual hemolysis occurring at that point. The curve is, in fact, a cumulative curve—known statistically as an *ogive*.

This being so, we can readily convert our findings in any case to indicate what degree of hemolysis occurs at any particular saline concentration.

All that is necessary is to deduct from the percentage hemolysis occurring at any particular level, that which occurred at the immediately higher concentration, and this will give the percentage hemolysis occurring in the range of saline concentration between these two points.

From the Royal Melbourne Hospital Melbourne, Victoria Australia.

The normal range of fragility as given by Creed<sup>2</sup> is shown in table 1 with its conversion to the derived curve. This is shown graphically in figure 1.

TABLE 1

Percentage of saline	0.28	0.32	0.36	0.40	0.44
Total maximal percentage of cells hemolyzed	100	98	90	46	10
Total minimal percentage of cells hemolyzed	98	90	45	10	0

Average saline concentration %	0.26	0.30	0.34	0.38	0.42	0.46
Maximal percentage of cells hemolyzed	0	2	8	44	36	10
Minimal percentage of cells hemolyzed	2	8	45	35	10	0

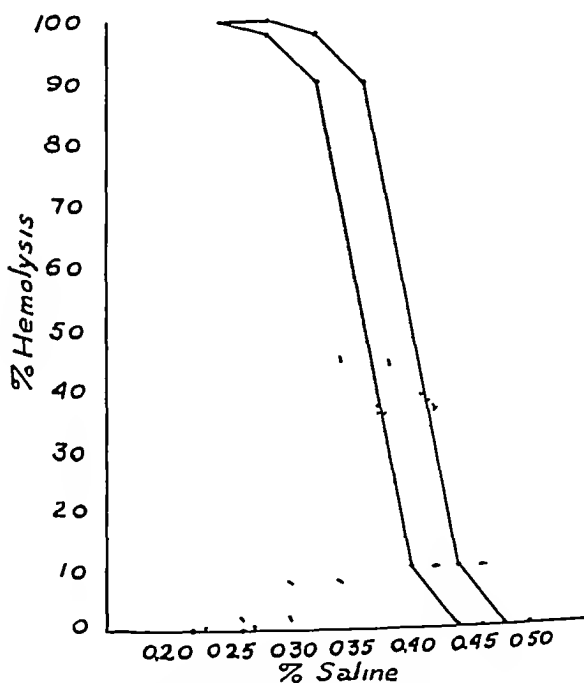


FIG. 1. Usual representation of normal range of fragility as sigmoid curves and the derived curves (dotted) of the distribution of red cell fragility.

The grouping is rather coarse but it will be seen that the two derived curves are very similar in form to that found in a Price-Jones curve.

If fragility were directly related to spherocytosis and spherocytosis only, this derived curve could be considered to be that of the distribution of cells in terms of their degree of spherocytosis, i.e., a curve directly comparable with a Price-Jones curve of diameter. Unfortunately, as pointed out by Ponder,<sup>18</sup> the red cell does

not act as a perfect osmometer, its degree of perfection in this respect being reduced by decrease in the tonicity of its environment. This is the probable explanation of the skewness of the curves shown for the normal distribution of hemolysis

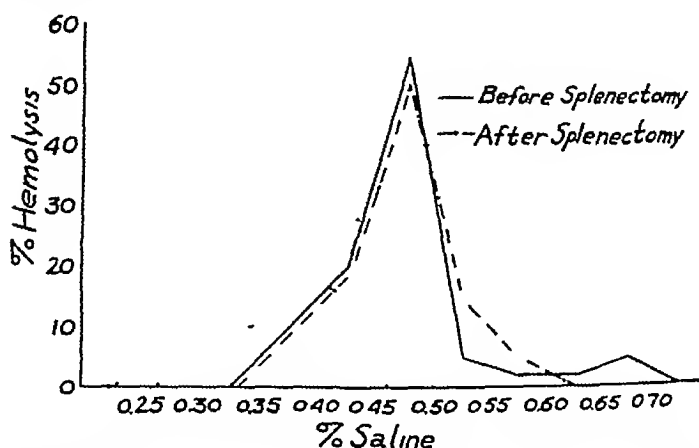


FIG. 2. Fragility distribution in a case of familial acholuric jaundice before and after splenectomy compared with the normal (dotted)

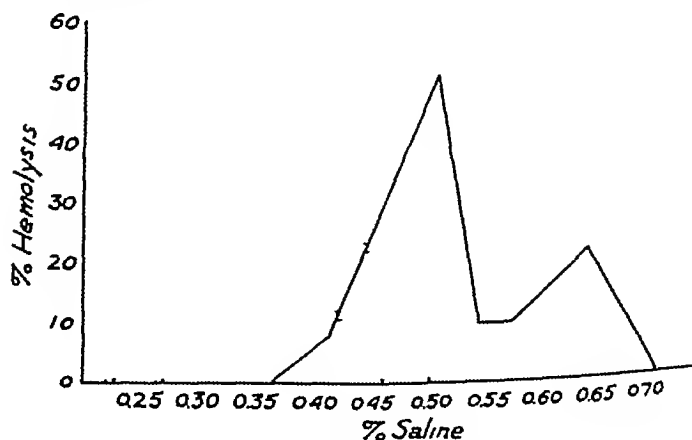


FIG. 3. Fragility distribution in a case of familial acholuric jaundice reported by Whitby and Hynes

#### APPLICATION TO CASES OF ANEMIA

Derived curves were calculated for various types of anemia

Figure 2 shows the findings in a case of familial acholuric jaundice before and after splenectomy. It will be noted that after splenectomy a smooth curve is obtained, abnormal in that its single mode occurs at a higher concentration of saline than normal. Before splenectomy the curve is not completely smooth but shows a second mode at a saline concentration of 0.65 per cent. This bimodal curve is seen again in a case described by Whitby and Hynes<sup>19</sup> (fig. 3) and suggests

that the cells are not homogenous but are composed of two populations of differing susceptibility to hypotonic saline

This aspect is further emphasised in a case of hypersplenism (fig 4) where we find, as might be expected, a maximal normal mode, but in addition, a marked secondary mode at a concentration of 0.45 per cent saline—a decidedly bimodal curve. Further examination of this curve shows that the abnormal peak involves

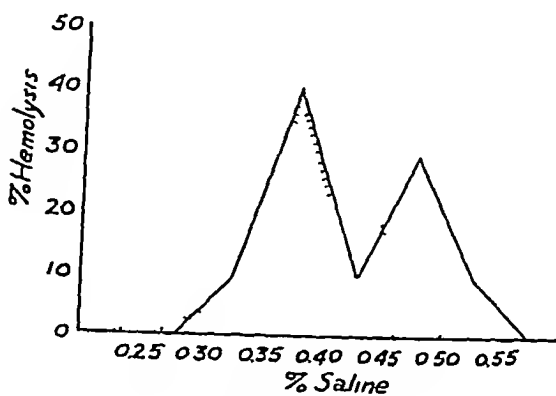


FIG 4 Fragility distribution in a case of congestive hypersplenism demonstrating marked bimodality

TABLE 2

Case	Hemolysis due to secondary mode	Reticulocyte
	%	%
R. & P		
D <sub>1</sub>	8	5.4
D <sub>2</sub>	7	16.0
R. <sub>2</sub>	5	9.6
M. <sub>1</sub>	20	26.0
D <sub>1</sub>	6	23.4
B <sub>1</sub>	10	11.0
B <sub>U</sub>	50	30.0
C. <sub>1</sub>	10	6.0
C. <sub>2</sub>	10	9.6
R. <sub>1</sub>	0	0.0
N	0	0.0
	0	0.0

some 30 per cent of the cells and this corresponds to the reticulocyte level at that time

Examination of other biphasic curves shows a similar correlation between the height of the secondary mode and the reticulocyte level. The correlation is even closer if, instead of taking the height of the secondary mode, we calculate the percentage of cells involved in that mode. This can be done only approximately, but gives a rank correlation of 0.67 per cent.

A case of acute hemolytic anemia reported by Ross and Paegel<sup>16</sup> is interesting

in that it shows three modes (fig 5) Reticulocyte counts and spherocyte counts were obtained on the same day, and their percentage corresponds roughly with the percentage of hemolysis involved in each particular modal area

A similar curve can be derived from the results of Goldbloom and Gottlieb,<sup>9</sup> who were investigating normal umbilical cord blood By examination of the residual cells after hemolysis, they showed that the early peak corresponded to disappearance of reticulocytes and the middle peak to destruction of nucleated red cells

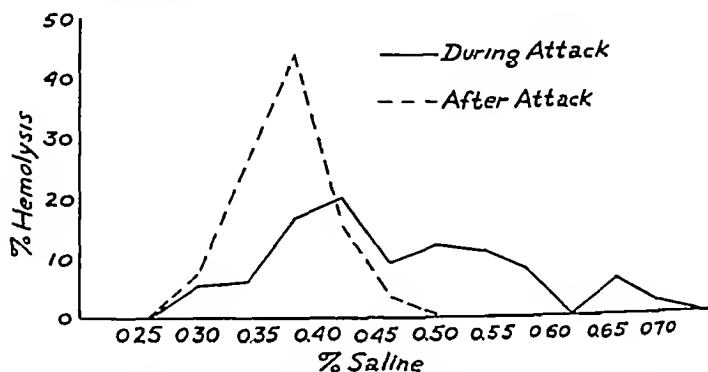


FIG 5 Fragility distribution in a case of acute haemolytic anaemia following sulphadiazine sensitization reported by Ross and Paegel Note the three separate modes during the attack and the single mode on recovery

TABLE 3

Reticulocytes	5.4%	Early mode	8%
Spherocytes	46.8%	Middle mode	40%

### DISCUSSION

Erythrocyte fragility has been expressed in different ways by different authors. It may be expressed as the range of saline concentration within which hemolysis occurs and notice may be taken particularly of the point where hemolysis is first seen—minimal fragility—or the point where hemolysis is complete—maximal fragility.<sup>13</sup> More commonly it is shown by means of a diagram relating percentage hemolysis to percentage of saline as previously described. All these methods are open to the criticism that they deal only with the extreme variability of the phenomena and pay little attention to the form of variation.

Various attempts have been made to obtain a single expression for fragility and the most satisfactory discussion on the subject is that of Janet Vaughan.<sup>14</sup> She used the median as her descriptive statistic and recognized that the sigmoid curve was an integral of cell fragility at specific levels. Unfortunately, she assumed that the curve was normal in type and on this basis attempted to express the variability as the slope (b) of the regression line of hemolysis (in terms of the standard deviation from the median) on saline concentration. Extreme values were neglected and

not surprisingly, she found this second statistic of little practical importance. A similar approach was used by Hunter,<sup>11</sup> who also realized that more than one maximum might be found, but was unable to explain this. His methods were used by Parpart et al.,<sup>14</sup> but uncritically in that a 'normal' curve was assumed.

From the previous results it will be seen that the curve of fragility is unlikely to be normal and in pathologic conditions frequently shows two or more modes. In the presence of such irregular curves the use of mean, median, or range, can lead to very erroneous conclusions and the advantage of the suggested method of presentation is that it permits the form of the distribution to be determined before using any statistic to describe position.

The apparent relationship between secondary modes and reticulocytes does not necessarily mean that reticulocytes are more fragile than normal. The presence of reticulocytes may only indicate increased marrow activity and this may be associated with the production of abnormally fragile cells quite apart from reticulocytes.

The literature on the subject of reticulocyte fragility is confusing, it being variously alleged that reticulocytes are less fragile, equally fragile and more fragile than normal.

That young cells are more fragile than normal appears to have been conclusively demonstrated by Cruz et al.<sup>2</sup> They used dogs rendered anemic by bleeding and tagged transfused red cells with radioactive iron so that their age could be followed. A marked difference in the fragility between young and old cells was found and this difference was maintained for five days. Thereafter the fragility of the old and the new cells became virtually identical. Reticulocyte counts were not done so that no further conclusions can be drawn with regard to these particular cells.

Key<sup>12</sup> concluded from his work that reticulocytes were normally fragile. He used rabbits as an experimental animal and emphasized the difficulty in performing reticulocyte counts after partial hemolysis. He pointed out that 'ghost' cells would still retain their reticulum which would stain and cause confusion in counting and also noted the danger of counting only the sedimented cells at the bottom of the tube. If this were done reticulocytes would appear to be more resistant than normal, but if samples were taken from the bottom of the tube and from the cells floating free in the plasma no constant differences could be obtained. This technique is not well described but, from the above description appears to be inadequate.

Swjatskaja<sup>17</sup> concluded that reticulocytes were less resistant than normal but his views were based on an apparent correlation between reticulocyte counts in the peripheral blood of anemic dogs and changes in the fragility of the cells. Residual cells were not examined for reticulocytes and his curves do not correlate accurately in time. The increase in resistance could have been due to the presence of target cells which, according to Bohrod<sup>1</sup> appear shortly after hemorrhage and are more resistant to hypotonic saline.

The most thorough examination of the problem was performed by Goldbloom and Gortlieb<sup>9</sup> who did reticulocyte counts and further fragility tests on the residual cells in each tube after a fragility estimation. Their work was performed on normal infant's blood from the umbilical cord and their conclusion was that reticulocytes were more fragile than normal.

Further work on this point is indicated.

In the application of these curves to actual cases of anemia, it would appear possible to decide whether an apparent increase in fragility was due to the result of an active bone marrow with the production of more fragile cells or to an intrinsic defect in some or all of the cells present. In this way the test should become enhanced in its diagnostic value.

## SUMMARY

- 1 A simple method of representing erythrocyte fragility as a distribution curve of actual cellular fragility is described
- 2 The importance of deciding the form of a distribution before using summarizing statistics is emphasized
- 3 The danger of concluding that abnormal fragility is present in the presence of increased marrow activity is pointed out
- 4 Possible applications in diagnosis are suggested

## REFERENCES

- <sup>1</sup> BOHRD, MILTON G The significance of target cells in anemia *Am J M Sc* 222 869 1941
- <sup>2</sup> CRUZ, W O HAHN P F, BALE W F, AND BALFOUR, W M The effect of age on the susceptibility of the erythrocyte to hypotonic salt solutions *Am J M Sc*, 222 157, 1941
- <sup>3</sup> CREED, E 10 *J Path and Bact*, 46 331, 1938 Quoted by WHITBY, L AND BRITTON C J C Disorders of the Blood 5th ed, London J & A Churchill 1947
- <sup>4</sup> DACIE, J V AND VAUGHAN J M The fragility of the red blood cells Its Measurements and Significance *J Path and Bact*, 46 341, 1938
- <sup>5</sup> DAMESHEK, WILLIAM AND MILLER EDWARD B Pathogenetic mechanisms in hemolytic anemias *Arch Int Med* 72 1 1943
- <sup>6</sup> — AND SCHWARTZ, S O The presence of hemolysins in acute hemolytic anemia *New England J Med*, 218 75 1938
- <sup>7</sup> — AND — Acute hemolytic anemia, (Acquired hemolytic icterus acute type) *Medicine* 21 231, 1940
- <sup>8</sup> —, AND NEBER J The improved demonstration of circulating antibodies in hemolytic anemia by the use of a bovine albumin medium *Blood*, 2 371 1947
- <sup>9</sup> GOLDBLOOM ACTON, AND GOTTLIEB, RUDOLF Icterus neonatorum *Am J Dis Child*, 39 57, 1949
- <sup>10</sup> HADEN R L The mechanism of the increased fragility of the erythrocyte in congenital hemolytic jaundice *Am J M Sc* 188 441, 1934
- <sup>11</sup> HUNTER F T A photo-electric method for the quantitative determination of erythrocyte fragility *J of Clin Investigation* 19 691, 1940
- <sup>12</sup> KEY J ALBERT Studies on erythrocytes with special reference to reticulum polychromatophilia and mitochondria *Arch Int Med* 48 511, 1921
- <sup>13</sup> MILLER E B, SINGER KARL, AND DAMESHEK WILLIAM Experimental production of target cells by splenectomy and interference with splenic circulation *Proc Soc Exper Biol & Med* 49 42, 1942
- <sup>14</sup> PARPART A K LORENZ P B PARPART E R GREGG S R AND CHASE, A M The osmotic resistance (fragility) of human red cells *J Clin Investigation*, 26 636 1947
- <sup>15</sup> PONDER, E The spherical form of the mammalian erythrocyte III Changes in surface area in discs and spheres *J Exper Biol* 14 267 1937
- <sup>16</sup> ROSS J F, AND PAROEL, BERTHA L Acute hemolytic anemia and hemoglobinuria following sulphadiazine medication *Blood* 1 189 1946
- <sup>17</sup> SWJATSKAJA, A Experimentelle Untersuchungen Über Erythropoese II Mitteilung *Ztschr f d exper Med* 52 150 1926
- <sup>18</sup> VAUGHAN, JANET M Red cell characteristics in acholic jaundice *J Path & Bact* 45 561 1937
- <sup>19</sup> WHITBY L E H, AND HYNES, M in *The Journal of Pathology & Bacteriology*, 46 219 1935 Quoted by WHITBY, L, AND BRITTON C J C Disorders of the blood, 5th ed London J & A Churchill 1947

# THE LABORATORY DIAGNOSIS OF SICKLE CELL ANEMIA WITH SPECIAL REFERENCE TO THE DIAGNOSTIC PARAMETER

By HERBERT M. PERR, M.D.

## INTRODUCTORY REMARKS

SICKLE cell anemia has been recognized as a specific entity since 1910 when it was described by Herrick.<sup>18</sup> Within thirteen years, 18 cases had been reported in the literature, and the clinical and hematologic characteristics had been almost completely described. This disease, defined as a hereditary, familial, acute and chronic hemolytic anemia,<sup>20, 21</sup> occurs predominantly in the Negro race,<sup>6, 13, 20, 23, 26</sup> and is one in which abnormally-shaped elongated erythrocytes can be demonstrated in vitro in the blood. This disease occurs in 0.18 per cent of the Negro population. The problem of early diagnosis assumes greater importance since it has been estimated that there may be as many as 135,000 cases of sickle cell anemia in this country.<sup>25</sup> It is also possible to artificially induce the presence of meniscocytosis,<sup>22</sup> drepanocytosis,<sup>14, 15</sup> and sickling in as many as 4-14 per cent<sup>4, 7, 12, 14, 16</sup> of Negroes tested without the presence of active sickle cell anemia. The etiology of this disease, transmitted as a Mendelian dominant, has not been satisfactorily explained. It is felt that the character of the erythrocyte<sup>25</sup> renders it more susceptible to hemolysis, and in great part, the clinical and pathologic changes are indicative of this process. In most cases, the diagnosis is obvious, but since sickle cell anemia can mimic rheumatic fever, other hemolytic anemias, polyarthritides, osteomyelitis, typhoid fever, pericarditis, catarrhal jaundice, meningitis, peptic ulcer, appendicitis, cholecystitis and other acute surgical abdominal conditions, the diagnosis in some cases depends upon adequate laboratory procedures.<sup>3, 6, 8</sup>

Until recently, the in vitro demonstration of sickled cells was considered the sole pathognomonic evidence of the trait. It was an early finding that the erythrocytes in a drop of capillary blood sealed from the air between a glass slide and a cover slip by liquid petrolatum<sup>10</sup> underwent progressive changes from the normal biconcave disc through various stellate and spiculated forms to a thin elongated type that resembled sickles.<sup>17</sup> Most workers agreed that the changes were due to the progressive oxygen unsaturation with the formation of reduced hemoglobin in the preparation.<sup>15, 25</sup> While it was possible to produce sickling in sealed preparations, this trait was frequently absent in simultaneous preparations of the same blood.<sup>1, 11</sup> In 1928, Hahn<sup>14</sup> noted that the formation of sickle cells under a cover slip is notoriously capricious, and Brandau<sup>4</sup> stated that in a given case, examination (wet smear) may be positive for the sickle cell trait at one time, at another negative. Some workers were able to demonstrate sickling in whole blood, and blood in various diluents<sup>8, 15, 19, 24, 27</sup> (1.25 per cent citrate in 1/8N sodium chloride, physiologic saline, serum from other patients), others were unable to reproduce these results.<sup>11</sup>

From Division of Laboratories, Newark Beth Israel Hospital, Newark, N. J.



In 1917, Hahn and Gillespie advocated the use of a small blood chamber in which carbon dioxide could be passed over a citrate suspension of blood.<sup>15</sup> In 1930, Scriver and Waugh suggested that a band be placed about the finger of an individual for five minutes before blood was drawn, the anoxic blood then demonstrating the trait more rapidly.<sup>20</sup> Hansen-Pruss,<sup>16</sup> in 1936, found that the addition of a drop of blood to a dried smear of one of several supravital stains (cresyl blue, janus green, or methylene blue) accelerated the rate of sickling. By this method, in 100 cases, 14 per cent were found to have the sickling trait, although the usually quoted figure is only half that amount.<sup>22</sup> In 1940, a comparison of the several methods of producing the trait (gas chamber, test tube, moist preparation, and the moist stasis preparation of Scriver and Waugh) revealed that the most reliable and the most practical method for the detection of the sickle cell trait is the moist stasis method of Scriver and Waugh.<sup>9</sup>

The differentiation between sickleemia and sickle cell anemia has been attempted frequently. In 1932, Diggs noted that sickle cells were seen more readily and in greater quantity in cases of sickle cell anemia than in instances of the sickling trait alone.<sup>7</sup> Sherman in 1940 proposed a method in which blood was collected anaerobically. In cases of sickle cell anemia, 30-60 per cent of the erythrocytes were sickled, while this was true in sickleemia to the extent of 1 per cent or less.<sup>17</sup>

A different approach to the laboratory diagnosis of this disease was made when the sedimentation rates were investigated. In 1927, Graham and McCarty noted that the erythrocyte sedimentation rate (E S R) seemed faster than normal,<sup>1</sup> a finding contradicted by later workers.<sup>4, 5</sup> In 1939, Bunting noted the fact that sickled erythrocytes from patients with sickle cell anemia did not form rouleaux, and in this condition did not sediment appreciably in one hour's time, whereas non-sickled forms from the same patients formed rouleaux and sedimented.<sup>8</sup> It was also noted that the uncorrected E S R may even be normal in the presence of marked anemia.<sup>2</sup> The characteristics of the E S R in sickle cell anemia were studied and it was noted that exposure to carbon dioxide caused sickling and a decrease in the E S R while oxygenation favored the development of rouleaux and accelerated the E S R. In 1944, Burch and Winsor investigated this property thoroughly and concluded that the variation in the E S R was a reversible procedure and could be controlled at will by exposing the blood either to carbon dioxide or oxygen.<sup>3</sup> From this and other data, a relationship between the differences in the E S R of blood saturated with carbon dioxide and oxygen was derived which was stated to be of diagnostic value in sickle cell anemia. In a study of 26 patients with sickle cell anemia and 60 patients who were normal or had diseases other than sickle cell anemia (371 determinations of which were done on the former and 239 on the controls), it was concluded that if the difference in the E S R's (called the diagnostic parameter or  $\Delta$ ) was 27 mm per hour or more, in 98 per cent of cases, sickle cell anemia would be present, and that the control subjects had 4 chances in much more than 10,000 of falling within the range for the sickle cell blood when the oxygen-carbon dioxide test is used.<sup>3, 9</sup> In the following year, the same workers reported their results when the diagnostic parameter had been in routine use on some services in Charity Hospital, New Orleans, for two years. In 61 consecutive

Negro admissions to one medical service, 44 per cent were found to have acute sickle cell anemia, a figure considerably higher than the usually quoted incidence. It was stated that in 437 control patients and 73 with sickle cell anemia, the diagnostic parameter did not give a false response in a single instance.<sup>30</sup>

Because of the reported accuracy and reliability of the test, and its simplicity and rapidity of performance, it was decided to test its application in a general hospital and to determine its practicability. It was hoped that during the evaluation of this test, previously unsuspected cases of sickle cell anemia would be discovered.

### METHOD

The blood specimens examined in this study were obtained predominantly from Negro outpatients attending the various clinics of the Newark Board of Health, Newark Beth Israel Hospital, and Newark City Hospital. In most cases the blood was obtained at the same time the routine serologic examination specimen was drawn.

The method described by Burch and Wiesor<sup>24-26</sup> was used with several minor modifications. Approximately 6-8 cc. of blood was drawn from a vein in the antecubital fossa and placed in a clean dry specimen bottle containing 15-25 mg. of sodium oxalate. The bottle was corked and rotated to insure thorough mixing. The relative proportion of blood to anticoagulant used does not seem to alter the E.S.R. appreciably.<sup>25-26</sup> Oxygen and carbon dioxide were introduced into separate 250 cc. Florence flasks and allowed to run in at the rate of 2-4 liters per minute for thirty to forty five seconds. A rubber stopper was then inserted into the mouth of the flasks. It was felt that the use of a Florence flask decreased the diffusion of the gas out of the container and that the larger size permitted a thinner layer of blood to be in contact with the gas over a larger surface area. About 2 cc. of blood was introduced into the flask by means of a graduated 5 cc. pipet and the flask rapidly sealed. This step usually took no longer than five seconds. The blood was allowed to remain in contact with the gas for about fifteen to twenty minutes during which the flask was rotated several times. The blood in the flask containing carbon dioxide turned a deep maroon in several minutes while that in the flask with oxygen assumed a bright scarlet color. No attempt was made to measure the degree of saturation by physicochemical means, the depth of color being sufficient indication. Before blood was placed in the Wintrobe sedimentation tubes the flasks were rotated so as to mix the blood thoroughly and render it homogeneous. The sedimentation tubes were tightly capped and suspended in a specially constructed rack. All readings were taken after one hour.

Concomitant sickle cell preparations were made. For the first 163 determinations, hanging drop preparations of oxalated whole blood in isotonic saline were used. Later it was felt that this method was not as accurate as the moist stasis method and accordingly moist stasis preparations<sup>32</sup> and wet sealed preparations of oxalated venous blood exposed to carbon dioxide were utilized. The latter, as previously described,<sup>32</sup> proved to be more accurate and was therefore adopted for the last 144 determinations.

All cases of sickling and high parameters were repeated whenever possible and all necessary clinical and laboratory data were obtained.

### RESULTS

Three hundred and seven determinations were performed upon 250 patients. Ten patients (4 per cent) had a diagnostic parameter of 27 mm. per hour or greater (table 1). One of these cases had acute sickle cell anemia, 7 had sickle cell anemia without anemia, and one case did not have sickle cell anemia. The remaining patient also demonstrated no sickle cell anemia, as evidenced by the relatively unreliable hanging drop method (it was not possible to recall this patient for a hemogram and a more accurate sickling preparation).

Fifteen patients (6 per cent) exhibited the sickling trait, and only one of these had acute sickle cell anemia. Of the 15, 8 patients had a diagnostic parameter

TABLE 1—Cases with high diagnostic parameters

Case	#*	E.S.R. mm/hr †		Δ	Sickling	Hb	RBC	WBC	Hist
		O <sub>2</sub>	CO <sub>2</sub>						
16	5	49	19	30	neg	%	million		
86	6	36	5	31	+	92	4.7	24,750	neg
95	9	46	2	44	+	86	4.25	6,550	—
101	5	38	0	38	—	79	4.24	6,250	—
121	7	46	1	45	+	—	—	—	—
138	2	46	1	45	+	94	4.74	7,250	—
150	3	59	1	58	+	34	1.34	44,250	+
191	3	32	1	31	+	82	4.32	7,700	—
224	3	32	5	27	+	80	4.03	7,050	—
224	2	43	4	39	+	72	3.80	10,350	luc
249	2	50	1	49	+	86	4.08	7,250	—

\* Number of determinations performed.

† Average value of results.

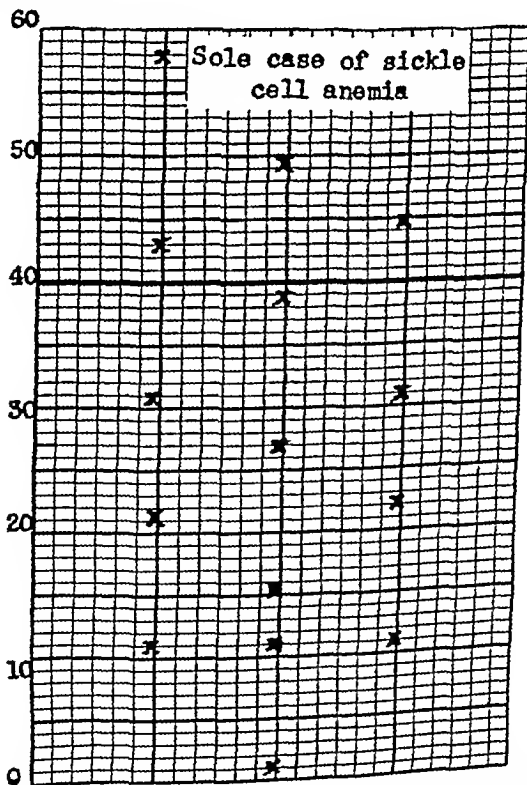


FIG. 1—DISTRIBUTION OF THE DIAGNOSTIC PARAMETERS OF 15 INDIVIDUALS WITH SICKLING

TABLE 2.—Comparison of Successive Readings Upon the Same Patient

Case	Date	Test No	Sed rate		$\Delta$	Sickling	Hb	RBC	WBC	History of S.C.A.
			O <sub>2</sub>	CO <sub>2</sub>						
15	2/12	17	48	34	14	*neg	%	million		
	2/13	21	46	32	14	*neg	66	3 53	3,900	neg
86	4/1	98	36	1	35	pos				
	4/10	125	36	1	35		90	6 80	4,250	neg
		126	36	1	35					
		127	37	1	36					
		128	37	1	36					
	5/6	204	36	24	12	pos	86	4 25	6,550	
	Average		36	5	31					
95	4/3	107	45	0	45	*neg	76	4 45		
	4/17	146	33	1	32	*neg				
		147	39	0	39					
		148	36	0	36					
		149	34	1	33					
	5/1	184	51	0	50	pos	79	4 24	6,350	neg
		185	54	2	52					
		186	52	1	51					
		187	54	15	39					
	Average		46	2	44					
101	4/4	113	37	0	37	*neg				neg
	4/24	170	37	0	37					
		171	43	0	43					
		172	37	0	37					
		173	37	0	37					
	Average		38	0	38					
106	4/8	118	9	6	3	*neg				
	4/17	150	14	9	5	*neg				
		151	14	10	4					
113	4/10	129	18	11	7	*neg				
		130	20	9	11					
114	4/10	131	15	6	9	*neg				
		132	13	5	8					
121	4/15	139	43	1	42	*neg				
	4/24	168	51	0	51					
		169	50	0	50					
	5/1	188	47	1	46	pos				
		189	44	0	44					
		190	43	2	41					
		191	45	2	43					
	Average		46	1	45					

\* Hanging drop preparation

above 27 mm per hour and 7 were below that value (fig 1). The diagnosis of sickle cell anemia was satisfactorily excluded in 14 of the 15 patients by the absence of anemia, jaundice, attacks of abdominal pain or arthralgia, pretibial ulcers, or on the laboratory findings of normal erythrocyte counts, hemoglobins (Sahli method), and total white counts.

For the most part it was noted that repeated determinations upon the same individual were in close agreement. In case 15, the values for the differential sedimentation rates obtained on two successive days were equal (table 2). In cases 101, 106, 113, 114, and 121, numerous determinations showed a high degree of correlation (table 2). However, in cases 86 and 95, a variation in results appeared. In the former, the first five determinations were almost exactly similar. The sixth, performed a month later, exhibited a marked increase in the ESR of the blood exposed to the carbon dioxide. This was difficult to ascribe to the technic as the method was standardized and relatively simple to perform. The possibility that a qualitative difference existed in the blood at the later date can not be excluded. Case 95 was similar (table 2).

### DISCUSSION

A high diagnostic parameter was associated with the presence of sickle cell anemia in almost all cases. There might have been a perfect correlation if the blood of case 101 (table 1) had been examined by a more sensitive sickling test than the hanging drop method. The single case of sickle cell anemia examined in this study had a diagnostic parameter above 27 mm per hour. Only 50 per cent of the cases of sickle cell anemia without sickle cell anemia had a parameter of 27 mm per hour or more. One might conclude that the diagnostic parameter, while highly indicative of the presence of sickle cell anemia, does not offer a means of differential diagnosis between sickle cell anemia and sickle cell trait.

### REFERENCES

- <sup>1</sup>ANDERSON, H. B. Sickle cell anemia. *Am J M. Sc.* 171: 641, 1926.
- <sup>2</sup>BAUER, J. Sickle cell disease. *Arch Surg* 41: 1344-1362, 1940.
- <sup>3</sup>— AND FISHER, L. J. Sickle cell disease. *Arch Surg* 47: 553-563, 1943.
- <sup>4</sup>BRANDAU, G. M. Incidence of sickle cell anemia trait in industrial workers. *Am. J. M. Sc.* 110: 813-817, 1930.
- <sup>5</sup>BUNTINO, H. Sedimentation rates of sickled and non-sickled cells from patients with sickle cell anemia. *Am J M Sc* 198: 191-193, 1939.
- <sup>6</sup>CANNY, C. B., CARPENTER, G. AND ELLMAN, L. Drepanocytosis (sickle cell anemia) and an apparently acute surgical condition of the abdomen. *Arch Surg* 48: 123, 1944.
- <sup>7</sup>DIGGS, L. W. The sickling cell phenomenon. *J Lab & Clin Med* 17: 913-920, 1932.
- <sup>8</sup>—, AND BIBB, J. The erythrocyte in sickle cell anemia. *J. A. M. A.* 112: 695-701, 1939.
- <sup>9</sup>— AND PETTIT, V. D. A comparison of methods used in the detection of the sickle cell trait. *J Lab & Clin Med* 35: 1106-1111, 1940.
- <sup>10</sup>EMMEL, V. E. A study of the erythrocytes in a case of severe anemia with elongated and sickle-shaped red blood corpuscles. *Arch Int Med* 20: 586, 1917.
- <sup>11</sup>GRAHAM, G. S. A case of sickle cell anemia with necropsy. *Arch Int. Med* 34: 778, 1924.
- <sup>12</sup>— AND MCCARTY, S. H. Notes on sickle cell anemia. *J Lab & Clin Med* 12: 536-547, 19-7.
- <sup>13</sup>GREENWALD, L., SPIELHOLZ, J. B., AND LITWINS, J. Sickling trait in a white adult associated with hemolytic anemia, endocarditis and malignancy. *Am J M Sc.* 116: 158, 1943.

- <sup>14</sup>HAHN E V Sickle cell (drepanocytic) anemia *Am J M Sc* 175 206-217, 1928
- <sup>15</sup>— AND GILLESPIE, E B Sickle cell anemia *Arch Int Med* 39 233-255 1927
- <sup>16</sup>HANSEN PRUSS, O C Experimental studies of the sickling of red blood cells *J Lab & Clin Med* 22 311-315, 1936
- <sup>17</sup>HEIN, G E McCALLA R L, AND THORNE, G W Sickle cell anemia *Am J M Sc* 173 763 1922
- <sup>18</sup>HERRICK J B Peculiar elongated and sickle cell shaped red blood corpuscles in a case of severe anemia *Arch Int Med* 6 517, 1910
- <sup>19</sup>HICK, J G Sickle cell anemia *Bull Johns Hopkins Hosp* 34 335, 1923
- <sup>20</sup>KRACKE, ROY Atlas of Hematology Philadelphia Lippincott 1941
- <sup>21</sup>MASON V R Sickle cell anemia *J A M A* 79 1318-1320 1922
- <sup>22</sup>MALLORY T Case records of the Massachusetts General Hosp #27421 *New England J Med* 225 616-630, 1941
- <sup>23</sup>MORRISON M, SAMWICK A A, AND LANDSBERG E Sickle cell anemia in the white race *Am J Dis Child* 64 881-887 1942
- <sup>24</sup>MURPHY, R C AND SHAPIRO S Sickle cell disease *Arch Int Med* 74 28-35, 1944
- <sup>25</sup>—, AND — The pathology of sickle cell disease *Ann Int Med* 23 376 1945
- <sup>26</sup>ROSENFELD S AND PINCUS J B The occurrence of sickle cell anemia in the white race *Am J M Sc* 184 674-782, 1932
- <sup>27</sup>SHERMAN I J The sickling phenomenon with special reference to the differentiation of sickle cell anemia from the sickle cell trait *Bull Johns Hopkins Hosp* 67 309-324 1940
- <sup>28</sup>WINSOR T AND BURCH, G E Rate of sedimentation of erythrocytes in sickle cell anemia *Arch Int Med* 73 41 1944
- <sup>29</sup>—, AND — Diagnostic physicochemical blood tests in sickle cell anemia *Am J M Sc* 107 152 1944
- <sup>30</sup>— AND — Sickle cell anemia a great masquerader *J A M A* 129 793, 1945
- <sup>31</sup>—, AND — Habitus of patients with active sickle cell anemia of long duration *Arch Int Med* 76 47 53, 1945
- <sup>32</sup>WINTROBE M M Clinical Hematology Philadelphia, Lea & Febiger 1946
- <sup>33</sup>—, AND LANDSBERG, J W A standardized technique for the blood sedimentation test *Am J M Sc* 189 101, 1935

# CONCOMITANT INFECTIOUS MONONUCLEOSIS AND HEMOLYTIC ICTERUS

By DAVID H. APPELMAN, M.D., and MAURICE M. MORRISON, M.D.

THIS COMMUNICATION describes a case of hemolytic anemia occurring in the course of infectious mononucleosis. A careful review of the literature reveals no precisely similar case.

Dameshek<sup>1</sup> reported an instance of hemolytic anemia in a patient with infectious mononucleosis who had been given sulfadiazine. The patient had cold hemagglutinins in his serum. Our case differs from that of Dameshek in that sulfonamide preparations had not been given, there was no preceding infection, and tests of the serum for cold agglutinins were negative. Ellis, Wollenman and Stetson<sup>2</sup> described a case of acute hemolytic anemia in an illness resembling infectious mononucleosis. Their patient was unlike ours in that tests of the serum showed auto hemagglutinins and hemolysins together with a positive Donath-Landsteiner reaction.

## CASE REPORT

A 22 year old, white single male was admitted to the Beth El Hospital on September 30, 1946. He had been well until five weeks before his admission, when he noticed weakness, fatigability, sluggishness and rust-colored urine. Jaundice and fever appeared in the fourth week of illness. He was hospitalized following a fainting spell. As a child he had experienced attacks of weakness followed by fainting spells. He underwent an appendectomy in 1943. Repeated bouts of furunculosis were successfully treated with penicillin while he was in Germany in 1945. No history referable to malaria or other hospital diseases was elicited.

On his admission the patient was slightly asthenic, with icteric skin and sclerae. The pharynx was injected, and small discrete lymph nodes were scattered throughout the neck. The spleen was palpable 4 cm. below the costal margin. Rectal temperature was 103 F. No other significant findings were noted.

Laboratory examinations on the morning following his admission were: hemoglobin, 4.3 grams (18 per cent), red blood corpuscles  $\times 4$  million, and 8,800 leukocytes per cu. mm. The differential count showed 70 per cent lymphocytes, 28 per cent segmented neutrophils and 2 per cent staff neutrophils. A few atypical lymphocytes characteristic of infectious mononucleosis were seen. About 25 per cent of the red blood corpuscles were spherocytic. A marked anisocytosis was present. Reticulocytes numbered 7 per cent. Five normoblasts per 100 W.B.C. were found on the blood smear. Platelets numbered 390,000 per cu. mm. Bleeding time, coagulation time, clot retraction time and prothrombin time were normal. A sternal marrow aspiration revealed a granulocytic-erythrocytic ratio of 50:50. M. g. erythrocytes were normal in number and the granulocytic elements were made up of 35 per cent myelocytes, 35 per cent segmented neutrophils, 10 per cent metamyelocytes and 20 per cent staff neutrophils.

The heterophile agglutination test was positive to a dilution of 1:512. The red blood corpuscle fragility test<sup>3</sup> with hypotonic saline solutions showed initial hemolysis at 0.48 per cent and complete hemolysis at 0.42 per cent. (With normal blood, hemolysis usually begins in the tube containing 0.44 or 0.4 per cent salt solution and is complete in the tube containing 0.34 per cent salt solution.) The urine was strongly positive for urobilinogen. Bile hemosiderin and hemoglobin were absent from the urine.

Blood chemical examinations showed an icterus index of 15, a delayed van den Berg reaction and a serum protein of 7.1 grams per cent. The albumin was 3.9 Gm. per cent and the globulin was 3.2 Gm. per cent. Serum total cholesterol was 200 mg. per cent and the cholesterol esters were 125 mg. per cent. Urea nitrogen, sugar and chlorides were normal. The cephalo flocculation test was 3 plus.

From the Departments of Medicine and Hematology, The Beth El Hospital, Brooklyn, New York.

The Kline test was negative. Blood cultures showed no organisms. Repeated fecal examinations failed to reveal any parasites or ova. No malarial parasites were found in the bone marrow and peripheral blood specimens. The patient was Rh positive and group O. Agglutination tests for brucellosis, typhoid, paratyphoid A and B, and typhus fever were negative.

Röntgenologic examinations of long bones and chest were normal. The basal metabolic rate was plus 18. Fragility tests done on the blood of the patient's relatives were within normal limits and no cytologic abnormalities were found.

The patient was given 3500 cc. of blood during the first week of hospitalization. The hemoglobin rose to 5.5 Gm. (36 per cent) with a red blood cell count of 1.9 million. The other hematologic and biochemical examinations were relatively unchanged. The urinary urobilinogen reached a titer of 1:100.

Two weeks after admission additional laboratory examinations were done: tests for cold agglutinins for paroxysmal hemoglobinuria by Mackenzie's modification<sup>4</sup> of the Donath-Landsteiner test, and for Marchiafava's disease by the Ham and Horack<sup>5</sup> procedure were all uniformly negative. The heterophile agglutination test was positive in a dilution of 1:256. The Davidsohn exclusion test confirmed the diagnosis of infectious mononucleosis.

The clinical course during the ten weeks of hospitalization was uneventful. The temperature returned to normal on the twelfth day, after reaching a peak of 104 F. forty-eight hours after admission. The lymphadenopathy persisted. The spleen became barely palpable. A slight icteric tint to the skin was still perceptible on discharge.

Laboratory examinations carried out during the last week of hospitalization showed a hemoglobin of 9 grams (58 per cent) with a red blood cell count of 2.8 million. Leukocytes were 7000 per cu. mm. of which 52 per cent were segmented neutrophils, 4 per cent eosinophils, 42 per cent lymphocytes and 2 per cent monocytes. No atypical lymphocytes were seen. A few macrocytes and an occasional spherocyte were seen on the stained blood smear. Reticulocytes were 3 per cent and the platelets numbered 380,000 cu. mm. Sternal marrow showed a granulocytic-erythrocytic ratio of 60:40. Granulocytic series showed 20 per cent neutrophilic myelocytes, 10 per cent eosinophilic myelocytes, 12 per cent metamyelocytes, 15 per cent staff neutrophils and 5 per cent eosinophils. The icterus index was 17. Quantitative serum bilirubin was 0.8 mgs. per cent. The heterophile agglutination test was negative. The red blood cell fragility test had returned to normal. Initial hemolysis began at 0.44 per cent and was completed at 0.32 per cent. The urinary urobilinogen was positive in a dilution of 1:100.

There was nothing in the patient's history prior to the onset of the infectious mononucleosis to indicate a pre-existing hemolytic disease. The subject was not seen until eighteen months after discharge. He looked well, had no icterus, and the spleen was not palpable. Laboratory examinations at this time were: hemoglobin 15.4 grams (100 per cent) red blood corpuscles 4.6 million and 6,000 leukocytes per cu. mm. The differential count showed 51 per cent segmented neutrophils, 10 per cent staff neutrophils, 33 per cent lymphocytes, 5 per cent monocytes and 1 per cent eosinophils. No pathologic cells were seen. Reticulocytes numbered 1.5 per cent, platelets 450,000/cu. mm. Bleeding and coagulation time were normal. The heterophile agglutination test was negative. The red blood corpuscle fragility test with hypotonic salt solution showed initial hemolysis at 0.44 per cent and complete hemolysis at 0.34 per cent. Blood chemical examinations showed an icterus index of 4.3 units, a negative cephalin-cholesterol flocculation test and a zinc turbidity test of 6.2 units. Bile hemoglobin and hemosiderin were absent from the urine. The urinary urobilinogen was within normal limits.

#### COMMENT

Following the report of the laboratory findings, it became apparent that the patient had hemolytic anemia and infectious mononucleosis concomitantly. The diagnosis of infectious mononucleosis was supported by the lymphocytosis with atypical lymphoid cells, the positive heterophile agglutination test, the lymphadenopathy and the splenomegaly. However the unusual findings were the presence of jaundice, anemia, an increased red blood cell fragility to hypotonic salt solution, spherocytosis, increased urinary urobilinogen and an erythroblastic marrow, i.e., the findings characteristic of hemolytic anemia. The occurrence of infectious mono-



nucleosis with jaundice has been previously described<sup>8,9</sup> Infectious hepatitis might be considered as a possible diagnosis but cases of infectious hepatitis almost invariably show an increased resistance to hypotonic saline Paroxysmal nocturnal hemoglobinuria was ruled out by a negative Ham acid test Reed and Helwig<sup>10</sup> reported 300 cases of infectious mononucleosis of which 3 presented severe anemia, but the anemia was associated with a marked reduction of the white blood cells and platelets The authors considered this pancytopenia to be part of the infectious mononucleosis

### SUMMARY

Hemolytic anemia concomitant with infectious mononucleosis, was observed in a 22 year old white male whose history indicates no pre-existing hemolytic disease Recovery occurred spontaneously

### REFERENCES

- <sup>1</sup>DAMESHEK, W Cold hemagglutinins in acute hemolytic anemia in association with sulfonamide medication and infection *J A M A* 123 77 1943
- <sup>2</sup>ELLIS L B WOLLENMAN J AND STETSON R P Autohemagglutinins and hemolysis with hemoglobinuria and acute hemolytic anemia in an illness resembling infectious mononucleosis *Blood* 1 419 1948
- <sup>3</sup>TODD, J C AND SANFORD A H *Clinical Diagnosis of Laboratory Methods* ed 10. Philadelphia, Saunders 1943
- <sup>4</sup>MACKENZIE, G M Observations on paroxysmal hemoglobinuria *J Clin Investigation* 7 27-43 1929
- <sup>5</sup>HAM G C AND HORACH H M Chronic hemolytic anemia with paroxysmal nocturnal hemoglobinemia *Arch Int Med* 67 735-745, 1941
- <sup>6</sup>BERNSTEIN A Infectious mononucleosis *Medicine* 19 85, 1940
- <sup>7</sup>BETHELL, F H, et al Blood A review of the recent literature *Arch Int Med* 74 131, 1944
- <sup>8</sup>CONTRATTO A W Infectious mononucleosis *Arch Int Med* 73 449 1944
- <sup>9</sup>STOLINSKY A Infectious mononucleosis A summary of the literature with a case report *Med Rec* 157 483 1944
- <sup>10</sup>REED J T AND HELWIG, F C Infectious mononucleosis An analysis of 300 cases with three characterized by rare hematological features *Arch Int Med* 95 376 1945

# INFECTIOUS LYMPHADENOSIS (MONONUCLEOSIS) AND HEMOLYTIC ANEMIA IN A NEGRO, RECOVERY FOLLOWING SPLENECTOMY

By SLOAN J. WILSON, M D, CHARLES E. WARD, M D, AND LUTHER W. GRAY, M D

IN MOST instances, infectious lymphadenosis (mononucleosis) is not a diagnostic problem. In a recent publication, Dameshek and Grassi<sup>1</sup> state that the lack of reduction of red cells and platelets in infectious lymphadenosis (mononucleosis) is of considerable aid in distinguishing this disease from acute lymphatic leukemia, and that the association of well-defined anemia and/or thrombocytopenia with a marked degree of lymphocytosis in which abnormal lymphocytosis is conspicuous almost certainly indicates acute leukemia.

These authors reported a case of severe thrombocytopenic purpura in a patient who had infectious mononucleosis. Splenectomy resulted in an excellent platelet response. The case herein presented is another exception to these generally accepted concepts in the diagnosis of infectious mononucleosis. A young Negro male was found to have generalized lymphadenopathy, slight hepatomegaly, marked splenomegaly, an absolute lymphocytosis and a severe anemia. Although the clinical picture strongly suggested leukemia, the qualitative characteristics of the lymphocytes were those of infectious mononucleosis and this diagnosis was confirmed by a markedly positive heterophile agglutination test. In addition to the marked anemia there was also a reticulocytosis, moderately increased red cell fragility, an elevation in the icterus index, and an increased urinary excretion of urobilinogen. This was interpreted as a hemolytic anemia. The family history was entirely negative. Splenectomy was performed when the anemia became extremely severe and uncontrollable by transfusions, and a prompt recovery occurred.

Infectious lymphadenosis is rare in the Negro, but has been reported.<sup>2-5</sup> Anemia has been reported with this disease in but a few cases.<sup>6</sup>

This case is of interest not only because of its rarity, but also because of the possibility of hypersplenism with resultant hemolytic anemia, thrombocytopenia and moderate leukopenia. The hemolytic anemia was the predominating feature of the disease.

## REPORT OF CASE

H, a young adult colored male, aged 18 years, was admitted to the hospital May 28, 1944. He complained of headache, fever and chills. A diagnosis had been made on May 23, 1944, of influenza. Because of a marked anemia with an erythrocyte count of 2.46 and hemoglobin of 6 Gm., he was admitted to the medical service (C. E. W.) for further study. The family history was entirely negative for hemolytic anemia.

On examination, the patient appeared to be acutely ill. The sclerae were jaundiced. The lymph nodes of the neck, axillae and inguinal regions were enlarged. A systolic murmur was present in the apical region. The liver was palpable at the costal margin. The spleen was enlarged and extended 8 cm. below the costal margin and medially to the midline.

On May 31, 1944, he was seen by one of us (S. J. W.). The physical findings were the same as on admission. Blood studies at this time revealed the following: erythrocyte count 2.46 M. h. = 21.4 G. Hb. 6.0

The patient was studied at La Garde General Hospital, New Orleans, Louisiana.

Gm (51 per cent), leukocyte count 19 500, reticulocytes 9.5 per cent, icterus index 17 units heterophile agglutination test positive to a dilution of 1:896 urinary urobilinogen positive in a dilution of 1:50. A red cell fragility test showed initial hemolysis at 0.45 and complete at 0.32 (normal initial hemolysis 0.41 complete 0.30). No sickling of red cells was observed in a 24 hour wet preparation. The platelet level by the Rees Ecker method was 240 000 per cu. mm. The peripheral blood was studied by the supravital technic and after having been stained with Wright's stain. The following differential count was obtained, polymorphonuclear leukocytes 20 per cent, lymphocytes 77 per cent, monocytes 1 per cent, eosinophiles 2 per cent. The lymphocytes varied in size and staining reaction and were typical of infectious mononucleosis. The erythrocytes showed some polychromatophilia, anisocytosis, and slight stippling. Spherocytes were numerous.

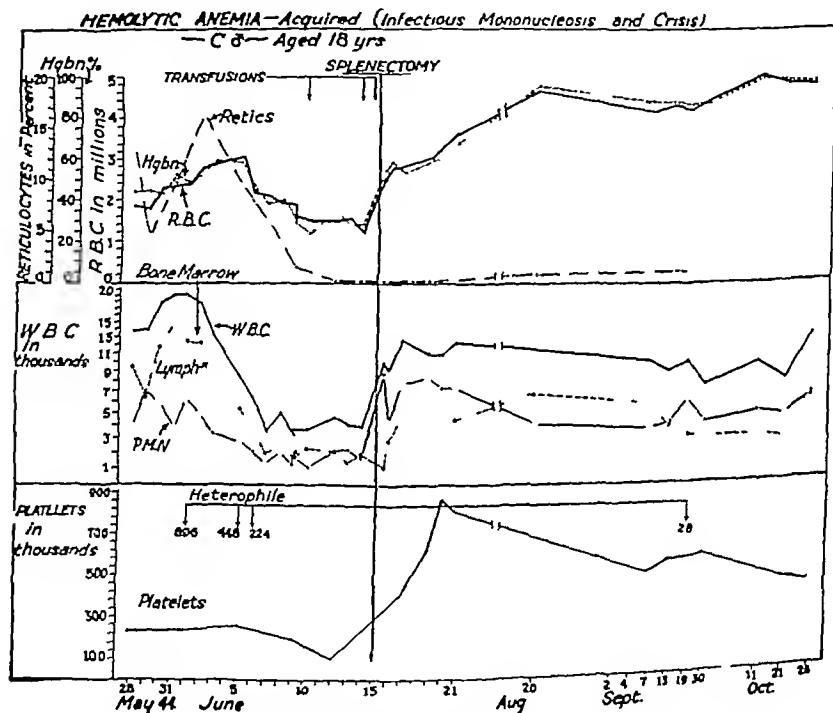


FIG. 1. Graphic illustration of the hematologic response in a Negro with infectious mononucleosis and hemolytic anemia. Splenectomy resulted in recovery.

A sternal marrow biopsy on June 2, 1944 revealed marked hyperplasia. Extensive plaques of erythroid elements at the normoblastic level of cell maturation were observed. The other marrow elements were normal in appearance and maturation levels but were relatively decreased. Occasional young lymphocytes were observed.

The patient began to improve clinically and hematologically (see fig. 1). A second hemolytic crisis developed however with a drop of erythrocytes, leukocytes and platelets. Transfusions of 500 cc. each were given on June 10, 14 and 15. The erythrocyte count on June 15 was 1.23 M. with a hemoglobin of 4.25 Gm (27 per cent). Because of the uncontrollable hemolytic phenomenon a splenectomy was done (L.W.G.) on June 15, 1944. The spleen was greatly enlarged and removed with some difficulty. Two accessory spleens were also removed. The red cell count in the afternoon of the operative day was 2.550 000 and hemoglobin 8.5 Gm (55 per cent).

The spleen grossly was markedly enlarged and moderately firm. The weight was 860 Gm. The capsule was smooth and not thickened. The splenic pulp was red and scraped with ease. Microscopic examination revealed marked dilatation of the sinusoids which were filled with erythrocytes. The malpighian bodies were not enlarged and the germinal centers were not proliferative in type. This probably is explained on the basis that the infectious mononucleosis had subsided before the splenectomy, as the lymph nodes had also receded.

The postoperative course was uneventful. The patient was observed until October 26, 1944. No recurrence of the hemolytic phenomenon was observed. On this date the following laboratory data were obtained: erythrocytes 4,950,000, leukocyte count 12,800 (higher than the usual periodic observations), hemoglobin 15.3 Gm (98 per cent), platelets 415,000, differential, polymorphonuclear leukocytes 48 per cent, lymphocytes 47 per cent, monocytes 1 per cent, eosinophiles 4 per cent, urinary urobilinogen slightly positive in a 1:5 dilution, heterophile agglutination positive 1:20 dilution.

### DISCUSSION

As has been stated before, infectious lymphadenosis is rare in the Negro, but has been reported.<sup>2-5</sup> It was quite evident in our case that there was a considerable mixture of negroid and white stock. Although negroid characteristics were present, the hair was auburn and the skin a very light brown.

Infectious mononucleosis is a readily recognizable disease, running a characteristic course. One of its outstanding features is the lack of anemia. Read and Helwig<sup>6</sup> reported anemia in only 6 of 300 cases of infectious mononucleosis. In 3 of these patients, there was a rapid and simultaneous drop in red blood cells, white cells and platelets. This was followed by a gradual rise in all three of the formed elements of the blood.

The 3 cases of Read and Helwig and the case reported here again serve to emphasize that a diagnosis between lymphatic leukemia and infectious mononucleosis cannot always be made on the absence of anemia and the presence of immature lymphocytes. The cells must indeed be studied for qualitative differences and correlated with the heterophile agglutination test. Read and Helwig, in addition to mentioning a possible hemolytic phenomenon, believed that the anemia may be partially explained by infiltration of the bone marrow. It is interesting to note that in one of their cases (case 3) the icterus index was 3.1 at the time the red blood cells were 1,900,000 and the indirect Van den Bergh test 3.5 mg. No other data are given which would be of aid to determine whether or not a hemolytic process existed.

In the case presented in this report, the cause of the hemolytic process cannot be definitely stated. No familial history could be obtained. The spleen was considerably enlarged and microscopically typical of a marked hemolytic process, the sinusoids being distended with erythrocytes. It could well be that this represented another manifestation of hypersplenism. Doan and Wright,<sup>7</sup> Doan<sup>8</sup> and Dameshek and Estren<sup>9</sup> have discussed at length the various types of hypersplenism of the primary and secondary types. Specific primary diseases exist in which an unstable splenic reticulo-endothelial system either acutely or chronically destroys and/or inhibits the blood cells, platelets and granulocytes excessively. These are descriptively identified as congenital hemolytic anemia, essential thrombocytopenic purpura, primary splenic neutropenia and primary splenic panhematopenia. One or other of these primary syndromes may be simulated by any secondary involvement of splenic tissue. Doan and Wright,<sup>7</sup> Doan<sup>8</sup> and Dameshek and Estren<sup>9</sup>

mentioned many such diseases in their discussions of the subject Hemolytic anemia however, is not mentioned as occurring in infectious mononucleosis One can hypothesize that in this case the spleen was stimulated to overdestruction of red cells and a severe hemolytic anemia resulted, which necessitated its removal As Dameshek and Grassi<sup>1</sup> state The whole subject of spleen-bone marrow relationships under normal and pathologic conditions has only recently come to the forefront, and many questions related to the possibly increased activity of the spleen must await further investigation

### CONCLUSION

A case is presented of acquired hemolytic anemia and infectious lymphadenosis (mononucleosis) in a Negro Splenectomy for the uncontrollable hemolytic state was followed by prompt recovery

### REFERENCES

- <sup>1</sup> DAMESHEK, WILLIAM AND GRASSI, MICHAEL A. Infectious lymphadenosis (mononucleosis) and thrombocytopenic purpura, recovery after splenectomy *Blood* 1 339 1946
- <sup>2</sup> LONGCOPE W T. Infectious mononucleosis (glandular fever) with a report of 10 cases *Am J M Sc* 164 781 1922.
- <sup>3</sup> RAY E S AND CECIL, R. C. Infectious mononucleosis in the Negro *South M J* 37 543 1944
- <sup>4</sup> JOHNSON ROSWELL D. Infectious mononucleosis in the Negro *J A M. A* 124 1254 1944
- <sup>5</sup> BLAIN ALEXANDER, 3RD, AND VONDER HEIDE, ELMORE C. Infectious mononucleosis and the Negro With a report of 6 cases *Am J M Sc* 209 587 1945
- <sup>6</sup> READ JOHN T AND HELWIG FERDINAND C. Infectious mononucleosis An analysis of three hundred cases with three characterized by rare hematologic features *Arch Int M-d* 75 377 1945
- <sup>7</sup> DOAN CHARLES A AND WRIGHT CLAUDE-STARR. Primary congenital and secondary acquired splenic panhematopenia *Blood* 1 10 1946
- <sup>8</sup> ——— Pan marrow hematopoiesis (L. casei factor) and splenic hematopenia Experimental and clinical studies *Proc. Institute Med of Chicago*, 16 No 6, June 15 1946
- <sup>9</sup> DAMESHEK, WILLIAM AND ESTREN SOLOMON. *The Spleen and Hypersplenism* New York Grunc & Stratton, 1947

# ABSTRACTS

JOSEPH F. ROSS, M.D., *Editor*

ABSTRACTERS

HELEN W. BELDING, M.D., Winston Salem, N.C.  
SHEILA T. CALLENDER, M.D., Oxford, England  
GEORGE E. CARTWRIGHT, M.D., Salt Lake City  
ROGER C. CRAFTS, Ph.D., Boston  
CHARLES P. EMERSON, M.D., Boston  
SOLOMON ESTREN, M.D., New York  
ROBERT S. EVANS, M.D., San Francisco

CLEMENT A. FINCH, M.D., Boston  
OLIVER P. JONES, Ph.D., Buffalo  
CONRAD MAIER, M.D., Zurich, Switzerland  
MILOŠ NETOUSEK, M.D., Prague, Czechoslovakia  
JEAN P. SOULIER, M.D., Paris  
RAMON M. SOAREZ, M.D., San Juan, Puerto Rico  
JAN WALDENSTRÖM, M.D., Uppsala, Sweden

## ERYTHROCYTES AND ANEMIAS

EFFECTIVENESS OF VITAMIN B<sub>12</sub> IN COMBINED SYSTEM DISEASE. RAPID REGRESSION OF NEUROLOGICAL MANIFESTATIONS AND ABSENCE OF ALLERGIC REACTIONS IN A PATIENT SENSITIVE TO INJECTABLE LIVER EXTRACTS. *L. Berk, D. Denny Brown, M. Finland, and W. B. Castle*. From the Harvard Medical School and the Thorndike Memorial Laboratory of Boston City Hospital, Boston, Mass., New England J. Med. 239: 328-330, 1948.

The authors report observations on a patient with pernicious anemia who was sensitive to pork and beef liver extracts. While taking folic acid she developed severe neurologic manifestations of combined system disease. She was then treated with 5 micrograms of Vitamin B<sub>12</sub> for eight days without reaction. Reticulocytes began to rise on the fourth day and reached a peak on the sixth day. By the tenth day neurologic regression was evident and the changes are tabulated by the authors. Discontinuance of B<sub>12</sub> for seven days led to some relapse which again responded to further treatment.

This report indicates that B<sub>12</sub> in contrast to folic acid should prove effective against the neurologic as well as the hematologic manifestations of pernicious anemia and that B<sub>12</sub> is not responsible for sensitivity reactions to liver extract.

C A F

OBSERVATIONS ON THE EFFECTS OF FOLIC ACID ANTAGONISTS. FOLIC ACID, LIVER EXTRACT AND VITAMIN B<sub>12</sub> ON EMBRYONATED EGGS. A PRELIMINARY REPORT. *P. F. Wagley and H. R. Morgan*. From the Thorndike Memorial Laboratory, Boston City Hospital and the Department of Medicine, Harvard Medical School, Boston, Massachusetts. Bull. Johns Hopkins Hosp. 83: 275-278, 1948.

Injection of folic acid antagonists produced a diminution in the size and number of the blood islets of the yolk sac of the embryo and degenerative changes in the nuclei of the islet cells. Of the three antagonists used, 4-amino-pteroylglutamic acid produced the most marked histologic changes. Injection of folic acid prior to administration of the antagonist did prevent the changes in the hematopoietic tissue but the preliminary injection of B<sub>12</sub> did not have an effect in the amounts used.

R S E

THE BLOOD AND BONE MARROW IN THE SPRUE SYNDROME. A STUDY OF 63 CASES. *E. M. Irwin*. From the Department of Medicine, University of Edinburgh. Edinburgh M. J. 56: 28-29, 1948.

Hematologic studies are recorded on a group of 27 adults with nontropical sprue, 17 adults with tropical sprue and 19 children with celiac disease. In the majority of adults macrocytic anemias were present and in those patients not under liver treatment, a megaloblastic marrow. Patients with celiac disease showed microcytic hypochromic erythrocytes. These studies are related to the morphology of erythrocytes and are somewhat difficult to interpret, since many patients were on therapy.

C A F

THE ANEMIA OF INFECTION. VII. THE SIGNIFICANCE OF FREE ERYTHROCYTE PROTOPHYLLIN IN THE ANEMIA OF INFECTION WITH SOME OBSERVATIONS ON THE MEANING OF THE EASILY SPLIT-OFF IRON. *M. Grollman and J. A. Grollman*.

and M. M. Wintrub From the Department of Medicine School of Medicine, University of Utah Salt Lake City J Clin Investigation 27 245-259 1948

In pursuance of earlier studies demonstrating an increase in erythrocyte protoporphyrin and urine coproporphyrin associated with the anemia of infection, experiments were designed to establish the significance of free protoporphyrin in red cells (EP) also included in the present report are observations relative to the nonhemoglobin iron, or easily split-off iron (ESFe) of the erythrocytes. Data obtained in the course of reticulocytosis produced in animals by hemolytic agents (employing phenylhydrazine and immune erythrocyte antibodies) and by restoration of deficiency anemias (pyridoxine deficiency in pigs and pernicious anemia in a human subject) and studies of effluent blood from congested spleens in nembutal treated animals indicated that the EP is greater and the ESFe less in immature than in mature red cells and that the erythrocyte EP in splenic venous blood was increased following splenic stasis.

These findings are interpreted as indicating that (a) an increase in EP usually signifies incomplete hemoglobin synthesis, as in reticulocytes in red cells altered by iron deficiency and those damaged by toxins or other factors or it may represent evidence of hemoglobin degradation, (b) the ESFe appears to be a degradation product of hemoglobin associated with the maturation destruction and perhaps senescence of red cells.

C.P.E.

#### VOLUME CHANGES IN HEMOLYTIC SYSTEMS CONTAINING RESORCINOL, TAUROCHOLATE AND SAPONIN E

Ponder From Nassau Hospital Mineola Long Island N Y J Gen Physiol 31 325-335 1948

Hemolysis produced by some lysins is preceded by a loss of potassium from the human red cell, and in the case of other lysins it may also be preceded by an increase in cell volume. A modification of the Hamburger (or van Allen) hematocrit method permitted the measurement of intact cells and the percentage of complete hemolysis. The results indicate that volume increases may be quite small while the potassium losses are larger, and that the volume changes may be unequal for equal potassium losses produced by different lysins.

O.P.J.

#### THE PERMEABILITY OF HUMAN RED CELLS TO CATIONS AFTER TREATMENT WITH RESORCINOL, N-BUTYL ALCOHOL, AND SIMILAR LYSINS E Ponder From Nassau Hospital Mineola Long Island N Y J Gen Physiol 32 53-62, 1948

In systems of washed cells of freshly drawn heparinized human blood to which various concentrations of resorcinol have been added, the loss of potassium increased with time. When potassium is made to re-enter cells which have previously lost it the quantity of sodium which leaves the cell is approximately the same as the quantity of potassium which enters.

O.P.J.

#### HYPOPHYSE ET HEMATOPOIESE I LE RETENTISSEMENT DE L'HYPOPHYSECTOMIE SUR L'HEMATOPOIESE DU RAT ALBINO (HYPOPHYSECTOMY AND HEMATOPOIESIS I THE EFFECT OF HYPOPHYSECTOMY ON HEMATOPOIESIS IN THE WHITE RAT L. Argy M. Gabe and F. Stumitsky Rev Hemat 3 154-179 1948

Twenty four male albino rats were hypophysectomized the weight and blood cell counts were followed and the bone marrow was examined. Histologic examinations using several techniques were utilized among them silver impregnation of the reticulum and detection of iron.

Some of the results are mere confirmation of what was already known namely anemia scarcity of erythroblasts in the marrow smears and splenic atrophy with increase of the lymphoid follicles.

Some points are of interest the effect on the bone marrow concerns not only the red cell series but also the myeloid series.

The osmotic fragility of the red cells in saline solution is decreased in the hypophysectomized rats. The increase in the lymphoid follicles in the spleen is nothing but a reflection of a general hyperplasia of the lymphoid tissues (lymph nodes Peyer's patches).

The study, using iron staining shows a striking hemosiderosis in the hypophysectomized animals. The authors believe that the bone marrow hyperplasia is linked to the thyroid atrophy which follows the hypophysectomy.

J.P.S.

**SULFHYDRYL COMPOUNDS AND THE SICKLING PHENOMENON** A PRELIMINARY REPORT *L Thomas and C A Stetson Jr* From the Department of Pediatrics, Johns Hopkins University Medical School and the Harriet Lane Home for Invalid Children Johns Hopkins Hospital Baltimore Maryland Bull Johns Hopkins Hosp, 83 176-180, 1948

The use of several reducing substances to produce rapid reduction in oxygen tension so as to promote sickling of susceptible cells is the subject of this preliminary report. Of the substances used, a saturated solution of hydrogen sulfide was the most active in producing sickling. Solutions of BAL and cysteine were also effective. After exposure to these substances the sickling phenomenon was found to be still reversible when the suspension was exposed to air. The concentrations of each substance necessary to produce sickling were also sufficient to produce a positive nitroprusside reaction.

R S E

**RAPID STAINING OF HEINZ BODIES IN SMEARS** *S H Webster E J Leljegren and D J Zimmer* From Laboratory of Physical Biology National Institute of Health Bethesda Maryland Stain Technol 23 97-98, 1948

The authors have used with equal success either a 0.2 per cent solution of methyl violet or crystal violet in 95 per cent ethyl alcohol. Freshly prepared air-dried moderately thick blood smears are covered with this solution for one half minute. The surplus dye is removed in running tap water.

O P J

**THE CYTOPLASMIC BASOPHILIA OF MARROW CELLS. THE DISTRIBUTION OF NUCLEIC ACIDS** *J N Davidson I Leslie and J C White* From the Department of Biochemistry St Thomas's Hospital Medical School London, England J Path & Bact 60 1-20, 1948

It has been shown by the application of Brachet's ribonuclease test and Caspersson's ultra violet absorption technic that young blood cells contain ribonucleic acid which diminishes progressively as the cells mature. The present study is an attempt to place some of these impressions on a quantitative basis. Marrow samples from 15 normal individuals and 22 suffering from various blood dyscrasias were prepared for studies of films, sections and chemical analysis. Quantitative determinations were made for total nucleic acid phosphorus, ribonucleic acid phosphorus and deoxyribonucleic acid phosphorus. The mean values of these for normal human marrow were 20.7, 14.2 and 6.9 mg of P per 100 Gm of fresh tissue respectively. These values were increased in hyperplastic immature marrows. Nucleic acid levels decreased during reticulocytosis in pernicious anemia following specific therapy and eventually returned to normal. If a hyperplastic marrow contains many cells of medium maturity, then the deoxyribonucleic acid phosphorus value is elevated. The high ribonucleic acid content of the cytoplasm and nucleoli of the younger marrow cells is apparently connected with the ability to pass through a series of mitotic divisions and to elaborate hemoglobin and specific granules. The nucleolus associated chromatin which increases as the nucleolar ribonucleic acid diminishes appears to persist after the cell has lost its power to divide.

O P J

**INTRODUCTION BIOLOGIQUE À L'ÉTUDE DES ANALOGUES DE L'YPERITE (SUBSTANCES DITES MOUTARDES À L'AZOTE OU AU SOUFRE) ÉTUDE CRITIQUE DES RÉSULTATS CLINIQUES OBTENUS LORS DU TRAITEMENT DE 40 HÉMATOPATHIES MALIGNES PAR UNE MOUTARDE À L'AZOTE. PREMIERS RÉSULTATS DE RECHERCHES BIOLOGIQUES EFFECTUÉES AU COURS DE L'ÉTUDE THÉRAPEUTIQUE DES ANALOGUES DE L'YPERITE (BIOLOGIC INTRODUCTION TO THE STUDY OF ANALOGUES OF TOXIC GASES [SUBSTANCES CALLED NITROGEN MUSTARD OR SULFUR MUSTARD] CRITICAL STUDY OF THE CLINICAL RESULTS OBTAINED DURING TREATMENT BY NITROGEN MUSTARD OF 40 MALIGNANT BLOOD DISEASES. FIRST BIOLOGIC RESEARCH RESULTS OBTAINED DURING THE THERAPEUTIC STUDY OF THE ANALOGUES OF TOXIC GASES)** *L Jastrow Besançon S Lamotte Barrillon and Cl Polonovski* Sem Hopit Paris 24 1511-1531, 1948

A complete historical and bibliographical review on mustard gas is given. The results of the authors' observations in 40 cases of malignant blood diseases, 19 cases of Hodgkins disease with nitrogen mustard are presented. The following conclusions were reached: Nitrogen mustard is often badly tolerated and



therefore it should be reserved for cases which can not be treated by x rays for practical reasons, and above all, for cases which become refractory to x rays. The very widespread forms are also more easily treated by nitrogen mustard but the results are usually of short duration. The importance of following hematologic changes is emphasized. Nitrogen mustard should not be given sooner than two months after radiotherapy. The results in the terminal cases of Hodgkins disease were disappointing.

The third part of this work is an experimental study of the methyl bis  $\beta$ -chloroethylamine in regard to the skin sensitivity, glucose and protein metabolisms, and antibody formation. In vitro the bactericidal activity of the drug was tested on several micro-organisms. The effect in vitro on the osmotic fragility of red cells on coagulation mechanism, and on different enzymes was also considered. Some derivatives, the ethyl instead of methyl and the brom instead of chloride, were tried in different diseases with good results. Finally, the prophylactic effect of hexamethylen tetramine against the toxic manifestations of the drug appears effective in rabbits and mice, and confirms the previous in vitro studies.

J.P.S.

**THE NATURE OF ANAEMIA IN LEUKAEMIA** D. H. Collins and W. McL. Ross. From Department of Pathology and Bacteriology, University of Leeds, England. *J. Path. & Bact.* 60: 63-74, 1948.

Fifty consecutive cases of leukemia were studied from 1943 to 1947. A significant anemia was present in every case of acute leukemia, in 75 per cent of the cases of chronic lymphatic and 65 per cent of the chronic myelogenous leukemias. The anemia of chronic lymphatic leukemia tended to be more severe at the time of diagnosis and later toward the end. Nucleated red cells appeared in the blood commonly in chronic myelogenous and acute leukemias. The author emphasized that, in the absence of icterus or osseous metastases, erythroblastosis in an adult with only moderate anemia should bring myelogenous leukemia to mind. In both lymphatic and myelogenous leukemia blood loss and destruction may aggravate the anemia. But in addition lymphatic leukemia has a hypoplasia of erythropoietic tissue through a crowding of the marrow by lymphocytes and myelogenous leukemia has a defective or disorderly erythropoiesis from a hyperplastic marrow. Some evidence has been presented to indicate that either pernicious anemia or a severe megaloblastic macrocytic anemia may precede the onset of acute leukemia.

O.P.J.

## BLOOD PIGMENTS

**METHEMALBUMIN. I. APPEARANCE DURING ADMINISTRATION OF PAMAQUINE AND QUININE** M. Reissfeld, C. G. Zubrod, W. D. Blake, and J. A. Shannon. From the Department of Medicine, New York University College of Medicine, and the Research Service, Third (New York University) Medical Division, Goldwater Memorial Hospital, New York City, and the Department of Pharmacology and Experimental Therapeutics, The Johns Hopkins University, Baltimore, Maryland. *J. Clin. Investigation* 27: 138-143, 1948.

Methemalbumin consistently appeared in the serum of individuals receiving antimalarial therapy with both quinine and pamaquine but did not complicate treatment with either drug when supplied alone or develop in patients receiving pamaquine and quinacrine concurrently.

A new and convenient procedure is described for the photometric determination of methemalbumin concentrations in serum utilizing an absorption band at 405 m $\mu$ . This method is applicable in the absence of hemoglobinemia and entails only the determination of the serum bilirubin concentration to obtain a factor for correction of the serum blank.

C.P.E.

**METHEMOGLOBINEMIA AND SULFHEMOGLOBINEMIA** C. A. Finch. From the Medical Clinics of Harvard Medical School and the Peter Bent Brigham Hospital, Boston, Massachusetts. *New England J. Med.* 239: 470-478, 1948.

The normal red cell mechanism for reducing methemoglobin and the ways in which this can be influenced are discussed. Methods of identifying methemoglobin and sulfhemoglobin clinical pictures associated with these pigments, etiologic agents in their production, and treatment are reviewed.

C.A.F.

activity in the hemophilic like blood and in combinations of the latter with normal blood demonstrated only a slight consumption of prothrombin in the course of the clotting process. It is concluded that the effect of this inhibitory agent was to impede the conversion of thromboplastinogen to thromboplastin. An explanation is thus afforded for the failure of certain patients with hemophilia and hemophilic like disorders to respond satisfactorily to transfusion therapy or the administration of antihemophilic globulin.

C P E

RELATION OF COMPLEMENT TO BLOOD COAGULATION *F D Mann and M Hurn* From the Division of Clinical Laboratories Mayo Clinic Rochester Minnesota *Proc Soc Exptl Biol & Med* 67 83-85, 1948

The role played by complement in the conversion of prothrombin to thrombin was studied by means of one and two-stage assays of thrombin production after recalcification and addition of thromboplastin in plasma freed of complement activity by aging by treatment with zymine and with ammonia. It was concluded that inactivation of complement by these methods prevents thrombin formation without significantly impairing the activity of prothrombin.

C P E

THE EFFECT OF HEPARIN AND DICUMAROL ANTICOAGULANT THERAPY UPON THE ERYTHROCYTE SEDIMENTATION RATE *S W Coggriff* From the Department of Medicine College of Physicians and Surgeons Columbia University and the Presbyterian Hospital New York City *J Clin Investigation* 27 435-438, 1948

The influence of anticoagulant therapy on the suspension stability of red cells was studied in 10 subjects receiving heparin 10 receiving dicumarol and in 5 recipients of both drugs concurrently. It was determined that in therapeutic dosages heparin and dicumarol do not significantly alter the erythrocyte sedimentation rate and that the results of this test are therefore not invalidated by interference from the effects of these drugs.

C P E.

## BLOOD PRESERVATION AND FRACTIONATION

BLOOD AND ITS DERIVATIVES *S T Gibson* From the Medical Clinic of the Peter Bent Brigham Hospital and the Department of Medicine Harvard Medical School, Boston, Massachusetts *New England J Med* 239 544-556 and 579-589 1948

This article with its bibliography of 381 references serves as an excellent review of the large amount of work undertaken during the war years on blood preservation and the uses of its various products.

Some of the general topics dealt with are blood preservation, reactions to blood products (especially serum hepatitis), procurement and fractionation of plasma therapeutic uses of albumin and other plasma components.

C A F

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## BOOK REVIEWS

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*Pathology of Nutritional Disease* By RICHARD H FOLLIS JR Springfield Ill C C Thomas 1948 pp 76

This is a beautifully printed and illustrated work in which the pathologic disturbances associated with nutritional deficiencies are described. The book is divided into six sections dealing with deficiencies of the essential elements: the essential amino acids, the fat and water soluble vitamins, and the pathologic anatomy of specific tissues. It contains superb illustrations both of gross and histologic material. There are

Twenty new born infants were found to have a high capillary resistance, about 50 centimeters of mercury. Among 16 premature infants, only 5 had a similar increased resistance, while 11 had a lower resistance, and 2 of the latter group (twins of 950 and 900 grams birth weight) had only 5 and 10 centimeters of mercury. The esculoside given to the mother during the labor seemed not to be effective on the capillary resistance of the child.

In conclusion, the authors believe that estrogen plays a great part in the capillary fragility of pregnancy and believe that the administration of folliculin to premature infants is not without danger.

J.P.S.

THREE-STAGE ANALYSIS OF BLOOD COAGULATION J. H. Milstone From Department of Pathology, Yale University School of Medicine New Haven Conn. *J. Gen. Physiol.* 31: 301-324, 1948.

The blood-clotting mechanism has been analyzed by a procedure which devotes a separate experimental step to each of the three primary reactions. The activation of prothrombin by thrombokinase followed the course of a bimolecular reaction. The activation of prothrombokinase involved an autocatalytic reaction.

O.P.J.

ACCELERATOR GLOBULIN AND ANTITHROMPHILIC GLOBULIN IN THROMBIN FORMATION FROM Aprotrombin AND IN HEMOPHILIC BLOOD J. H. Ferguson and J. H. Lewis From the Department of Physiology University of North Carolina, Chapel Hill North Carolina. *Proc. Soc. Exper. Biol. & Med.* 67: 228-231, 1948.

A series of *in vitro* experiments are reported designed to characterize more completely an accessory clot promoting factor variously designated as labile factor (Quick), factor V (Owren) and accelerator globulin (Ware, Guest and Seegers). This factor, present in fresh plasma apparently potentiates by some mechanism unrelated to the plasma protease system, the conversion of prothrombin to thrombin in the presence of active thromboplastin and the calcium ion. Tests conducted with a purified fraction of bovine plasma containing the factor demonstrated a loss of potency with aging; its deterioration under these conditions occurring independently of prothrombin inactivation. Applied in the fresh state however or after storage in the frozen state, this factor effectively restored the original activity of aged prothrombin preparations.

The factor is possessed of no antihemophilic properties, its action being unrelated to that of thromboplastin or any of its precursors or activators. A naturally occurring deficiency of this plasma factor is believed to be the basis of a specific bleeding disorder, Owren's disease, (*Lancet* 232: 446, 1947) to be distinguished from hemophilia, idiopathic hypotherbinemia and other hemorrhagic syndromes.

C.P.E.

ACTIVATION OF PLASMA THROMBOPLASTINOGEN AND EVIDENCE OF AN INHIBITOR A. J. Quick and M. Stefani From the Department of Biochemistry, School of Medicine Marquette University, Milwaukee Wisconsin. *Proc. Soc. Exper. Biol. & Med.* 67: 111-112, 1948.

The first reaction involved in the mechanism of blood clotting according to the authors is the enzymic conversion of the thromboplastic precursor thromboplastinogen, a normal plasma constituent, to active thromboplastin through the agency of a platelet factor. In hemophilia the clotting defect is related primarily to a deficiency of thromboplastinogen, the platelets in this disorder exhibiting normal clot promoting activity when added to deplateletized normal plasma. Evidence is cited (J. Clin. Investigation 25: 814, 1946 and *Science* 106: 473, 1947) indicating that the situation in hemophilia is occasionally complicated by the appearance of an inhibitory factor in the blood which imparts to the latter anticoagulant properties.

A case is described in which a hemophilia like disorder developed following pemphigus. The prothrombin activity assayed with serial dilutions of thromboplastin was normal; thus excluding the presence of an antithromboplastin. The clotting time of this patient's blood was markedly prolonged; moreover it was essentially unaltered by the addition of normal blood, hence, the abnormality was presumably not attributable to a deficiency either of thromboplastinogen (as in true hemophilia) or of the platelet factor. Since the clotting time of normal blood was delayed when mixed with the patient's blood it is assumed that a clot inhibitor was operative. Finally, since measurements of prothrombin

activity in the hemophilic like blood, and in combinations of the latter with normal blood demonstrated only a slight consumption of prothrombin in the course of the clotting process, it is concluded that the effect of this inhibitory agent was to impede the conversion of thromboplastinogen to thromboplastin. An explanation is thus afforded for the failure of certain patients with hemophilia and hemophilic like disorders to respond satisfactorily to transfusion therapy or the administration of antihemophilic globulin

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C.A.F.

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There are 791 references and 71 superb illustrations, both of gross and histologic material. There has

been need for such a carefully conceived work and the book is recommended highly to nutritionists, internists, and pathologists

WILLIAM DAMESHEK

*El Diagnóstico por la Punción Ganglionar* 1947 By G. FORTEZA BOVER. Valencia: Editorial Saber, 1948. In Spanish, 146 pages, 55 figures in black and white.

In referring to the history of lymph node puncture, the author points out that although it had been recommended as a means of diagnosis in isolated cases since the beginning of the century, it had come into general use only following the publications of Ellis and Martin (1930-1934) in the United States and of A. Pavlovsky of Buenos Aires (1933-34). Thereafter, well documented monographs by Lutrozu, Weill, Stabel, Tischendorf, Leitman and others appeared.

Utilizing the technique recommended by Pavlovsky, Forteza Bover presents his experience, beginning by describing in detail the cytomorphology of normal and hyperplastic lymphatic tissue. The disorders of lymphoid tissue are then described, beginning with the simple hyperplastic processes. There are excellent illustrative photomicrographs and colored plates.

In tuberculous adenitis, four stages are described: (1) initial tuberculous hyperplasia, (2) granulomatous transformation with necrosis and the presence of Langhans' giant cells with necrosis, (3) caseous necrosis and (4) purulent effusion. Careful descriptions of Boeck's sarcoid are given with figures illustrating the difficulties in differential diagnosis from tuberculous adenitis.

The author's studies of Hodgkin's disease are in agreement with those obtained by previous workers who have utilized similar diagnostic techniques. In lymphosarcoma, the cytologic characters of tumor formation are well depicted. However, in the diagnosis of follicular lymphoblastoma, the author considers the cytologic picture as being nonspecific and requiring an open biopsy for a definite diagnosis.

The pictures obtained in metastatic malignancy are of great value and Stabel's descriptions of the cellular elements which permit diagnosis are followed.

This book is well presented with good documentation and some excellent color plates. There are some original observations in Boeck's sarcoid and comprehensive and detailed studies of Hodgkin's disease. The book is recommended particularly to those who wish to become acquainted with the technique of and results obtained from lymph node puncture and study of the adenogram.

ALFREDO PAVLOVSKY

*New Staining Methods in Hematology* 1948 By J. GARCIA BLANCO AND G. FORTEZA BOVER. Valencia: Editorial Saber, 1948. In Spanish, 93 pages, 19 plates in color and 11 engravings in black.

The authors describe staining methods which were at first used histochemically but which were later applied to studies of the blood and bone marrow.

The various oxidase methods are described. The use of tetrabromophenolphthalein for staining hematopoietic tissues is described for the first time, used both singly and in combination with the peroxidase methods.

In the method which the authors call T.F.S. (Neosina), they utilize tetrabromophenolphthalein to retain the cellular structure of the various tissues. T.F.S. appears to react with the simple proteins and the prosthetic groups of the cellular structures, causing color combinations which depend upon the isoelectric point of the substrate. They also use the combined method with eosin and methylene blue with which beautiful contrast pictures are obtained.

This book is carefully and clearly written with exact descriptions of the techniques used and is exceptionally well illustrated. The excessive detail in the described techniques is somewhat confusing in the minutiae. For this reason, it is unfortunate that the authors did not complete their work by presenting a precise critique of the different staining techniques, thus advising which of the methods used is of greatest application in a given instance.

ALFREDO PAVLOVSKY

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## *Contributors to this Issue*

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**B ALEXANDER, M D**

Visiting Physician and Associate in Medical Research, Beth Israel Hospital, Associate in Medicine, Harvard Medical School, Boston, Mass

**Y M BHENDE, M D**

Assistant Professor of Pathology, Gordhandas Sunderdas Medical College Bombay, India

**WILLIAM T COLLINS, M D**

Formerly Lieutenant junior grade (MC) USNR Assistant Laboratory Officer U S Naval Hospital Oakland, Calif, now Instructor in Pathology, University of Cincinnati, Cincinnati Ohio

**H L D DUGUID, M B, Ch B**

Assistant in Medicine, University of Aberdeen, Aberdeen, Scotland

**H W FULLERTON, M D, M R C P**

Professor of Medicine, University of Aberdeen Physician, Aberdeen Royal Infirmary, Aberdeen, Scotland

**R GOLDSTEIN, M D**

Associate in Medical Research and Associate in Medicine, Beth Israel Hospital, Assistant in Medicine, Harvard Medical School, Boston, Mass

**J PHILIP LOGE, M D**

Department of Medicine Washington University Medical School, St Louis Mo

**J C PATEL, M D, Ph D, M R C P**

Honorary Physician, Singanee Hindu Hospital Honorary Assistant Physician, King Edward Memorial Hospital, Bombay, India

**JAMES A RILEY, M D**

Resident in Internal Medicine University of Oregon Medical School Hospitals and Clinics Portland Ore

**GEORGE M ROBINS, M D**

Clinical Instructor in Medicine University of Oregon Medical School Attending Staff, Emanuel Hospital, Portland, Ore

**R WAYNE RUNDLES, Ph D, M D**

Assistant Professor of Medicine, Duke University School of Medicine, Assistant Physician, Duke Hospital Durham N C

**MAX SAMTER, M D**

Assistant Professor of Medicine, University of Illinois College of Medicine, Chicago Ill

**A DE VRIES, M D**

Research Fellow in Medicine, Harvard Medical School, Boston, Mass

**HARRY A WEISS, M D**

Lieutenant (MC) USN Resident in Internal Medicine, U S Naval Hospital Oakland Calif Now at U S Naval Hospital, Corona, Calif

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# BLOOD

## *The Journal of Hematology*

VOL IV, NO 3

MARCH, 1949

### URETHANE (ETHYL CARBAMATE) THERAPY IN MULTIPLE MYELOMA

By J PHILIP LOGE, M D , AND R WAYNE RUNDLES, M D

INTEREST in the growth-suppressive properties of the carbamic esters, known for many years from general biologic studies, was renewed in 1945 by the investigations of Templeman and Sexton.<sup>1</sup> In studying the effect of ethyl phenylcarbamate on the growth of plant seedlings, they confirmed the earlier work of Lefèvre showing that this chemical retarded or even arrested plant growth, with various structures undergoing bulbous hypertrophy. Cytologically it appeared that mitosis was blocked in pseudometaphase, leading to the irregular formation of monstrous nuclei. Haddow and Sexton<sup>2</sup> then studied the effect of different carbamic esters on experimental animal tumors. They found that the common urethane, ethyl carbamate, was the most promising compound. It produced a transient increase in mitosis in normal tissues, and a significant retardation in the growth of spontaneous mammary cancer in the mouse and in the growth of the Walker rat carcinoma 256. The histologic structure of the tumors was profoundly altered.

Clinical trials using urethane and isopropyl phenylcarbamate in the treatment of advanced inoperable cancer were then undertaken. Amelioration was observed in a few cases but the results were generally disappointing. It was noted, however, that a fall in the leukocyte count occurred with some regularity. The experiments were then modified to include myeloid and lymphatic leukemia. Here the effects were vastly more pronounced, indeed comparable in many ways to those obtained by roentgen irradiation.<sup>3</sup> Following the report of Paterson, Ap-Thomas, Haddow, and Watkinson,<sup>3</sup> urethane began to be used extensively in the treatment of leukemias and widespread tumors in clinics throughout the world.<sup>4-11</sup>

The therapeutic limitations of urethane have become clearer as experience has widened. In disseminated cancer, in spite of an occasional success, there is usually no benefit from this therapy.<sup>5-9</sup> In localized lymphomas, roentgen irradiation remains the treatment of choice.<sup>14</sup> In acute leukemias, there is generally no improvement.<sup>4</sup> In chronic leukemias the net clinical benefit in many cases is probably less than that obtainable by standard methods of treatment. A disease in which exceptional therapeutic results may occur, however, is multiple myeloma, for changes have been observed following urethane administration which appear to be unique in the therapy of this disease.

From the Department of Medicine, Duke University School of Medicine and The Hematology Laboratory, Duke Hospital, Durham, North Carolina.

This study was aided by a grant from the Anna H. Hanes Fund.



The major features of multiple myeloma have been reviewed recently by Bayrd and Heck<sup>16</sup> and Lichtenstein and Jaffe.<sup>18</sup> It is a uniformly malignant disease progressing to a fatal termination in an interval varying from a few weeks to some times several years. The disease results essentially from the excessive proliferation of abnormal plasma or myeloma cells within the bone marrow. Anemia, leukopenia and at times thrombocytopenia result. Skeletal support becomes seriously impaired as the bones soften due to diffuse demineralization or to the formation of multiple areas of osseous destruction showing virtually no detectable attempt at repair. A complex abnormality of the body proteins is a feature of the disease being manifested by abnormally high serum protein values, hyperglobulinemia and often Bence-Jones proteinuria. Grave renal disease is a commonly associated finding.

Many different types of therapy have failed to alter significantly the clinical course of multiple myeloma. Roentgen irradiation, which has been used extensively, is fairly effective in relieving localized bone pain.<sup>17, 18</sup> Radioactive phosphorus, likewise, may relieve skeletal pain but even with doses large enough to produce leukopenia and thrombocytopenia there has been no overall improvement.<sup>19</sup> A few patients with multiple myeloma have been treated with nitrogen mustard compounds but the results have been disappointing.<sup>20-22</sup> Myeloma cells do not decrease in number in the bone marrow and there is no change in the histologic structure as observed in serial biopsies.<sup>23</sup>

Another approach to the chemotherapy of multiple myeloma was introduced by Snapper<sup>24, 25</sup> who used injections of stilbamidine and pentamidine combined with a diet low in animal protein. Symptomatic improvement and even bony recalcification was noticed in some patients. Myeloma cells persisted in the bone marrow without quantitative decrease, however, as did Bence-Jones proteinuria and hyperglobulinemia. Relapses occurred. Serious toxic reactions limited the usefulness of the diamidine compounds. Antimony compounds were used by Rubinstein<sup>26</sup> with similar results.

Reports of urethane therapy in patients with multiple myeloma are few. Paterson and her co-workers<sup>3</sup> observed no improvement after giving urethane to 2 patients with multiple myelomatosis. Berman and Axelrod<sup>9</sup> gave 72 Gm. of urethane to a 51 year old man with multiple myeloma but discontinued therapy when leukopenia developed. Hyperglobulinemia and Bence-Jones proteinuria was unchanged. Serial x-rays showed no change. Alwall<sup>27</sup> used urethane in the treatment of two patients with multiple myeloma. To one who complained mainly of skeletal pain he gave 3-4 grams of urethane daily for three months, without noticeable improvement. Stilbamidine was then injected intravenously and he was rather promptly relieved of pain. Other aspects of the disease were not affected. A second patient whose complaints related to anemia was given urethane alone and observed over a period of eight months. The anemia, albuminuria, hyperglobulinemia, and rapid sedimentation rate all improved considerably during the first four months of treatment and myeloma cells could no longer be found in the bone marrow.

This spectacular but isolated result suggested to us a more extensive and pro-

longed trial of urethane therapy in multiple myeloma. We have now studied the early therapeutic responses of 4 patients to urethane during observation and follow-up periods ranging from seven to thirteen months. Other types of specific therapy have been withheld. The detailed case histories follow.

TABLE I—Case 1 C F H, B-25150, Hematologic Findings

Date 1948	Hgb	RBC	WBC	Hemato- crit	Reticulo- cytes	Plate lets	Differential WBC and therapy	
	Gm per 100 cc.	mill per c mm			%	thou sands per c mm		
1/30	8.1	2.7	9750	25	1.6	1000	Neutrophils 42, stabs 8 metamyelo- cytes 6, myelocytes 3, myeloblasts 1, lymphocytes 30 monocytes 7 plasma cells 3 normoblasts 3/100 WBC.	
	Bone Marrow Plasma cells 98 neutrophils 1, monocytes 0.5 lymphocytes 0.5							
2/18	7.1	2.4	10950	21	2.7	480	Urethane 6 Gm./day	
2/21								
2/25	6.6	2.3	19000	20	1.6	316		
3/2	7.0	2.4	6150	21	1.5	530		
3/9	6.6	2.5	2900	22	4.2	560	Urethane 1.5-2.0 Gm./day (120 Gm.)	
3/16	7.0	2.8	2600	24	8.7	112		
3/26	6.9	2.66	3000	23	3.2	428		
4/9	9.3	3.1	3500	29	6.0	1330		
4/19	9.2	3.1	4900		6.9	601	Neutrophils 71 stabs 3 lympho- cytes 20, monocytes 6	
	Bone Marrow Plasma cells 1, neutrophils 9 stabs 16 metamyelocytes 9, myelocytes 10, myeloblasts 3 lymphocytes 2, mono- cytes 1, macrophages 1, reticulum cells 3, eosinophils 2, erythroblasts 5, basophilic normoblasts 15, acidophilic normoblasts 23							
4/23	11.0	3.9	6100	37	5.1	490		
5/21	11.1	4.3	5350	36	2.7			
6/4	11.8	4.3	8450	38	1.0	688		
6/18	11.5	4.1	4900	38	1.2	762		
10/19	14.0	5.05	6950	44.5	2.0			

## CASE REPORTS

**Case 1 C F H, Unit No B-25150.** This 41-year-old colored woman was referred to Duke Hospital on January 28, 1948. Her health had been good until six months earlier when she began to have chest pain when coughing or when pressure was exerted against her ribs. In the course of another two or three months she was forced to quit work because of pain along the spine and about her hips. The skeletal pain increased progressively. During her last month at home she was unable to walk or even get out of bed. She lost about ten pounds in weight.

Physical examination showed her to be a well-developed, well-nourished colored woman unable to move about on the examining table without acute discomfort. Pressure over the sternum, ribs, and spines of the vertebrae was exquisitely painful. The remainder of the examination disclosed no relevant abnormalities.

Examination of the peripheral blood showed that there was a severe anemia with immature erythrocytes, plasma cells, and nucleated red cells in the circulating blood (table 1). Rouleaux formation was

conspicuous in the blood films. Urinalysis showed a small amount of protein and a trace of Bence Jones protein. The excretion of phenolsulphonphthalein dye was not impaired. Serologic tests for syphilis were negative. The serum proteins were 13.2 Gm. per 100 cc. with 4 Gm. of albumin and 9.2 Gm. of globulin on one determination and 10 Gm. with 3.4 Gm. albumin and 6.6 Gm. globulin on another (table 5). The blood calcium was 12.4 Gm. per 100 cc., phosphorus 3.6 Gm., and alkaline phosphatase 3.1 Bodansky units per 100 cc. A bromsulphalein test of liver function, using 5 mg. of dye per kilogram of body weight showed 7 per cent retention after 45 minutes. Roentgen examination of the skeleton (fig. 1 A and B) showed pronounced generalized demineralization with small and large areas of non-reactive destruction in the skull, ribs, vertebrae, pelvis and long bones. There was partial collapse of the ninth and twelfth thoracic vertebrae.

Bone marrow was obtained by sternal aspiration. The bone was so soft that there was almost no resistance to insertion of the needle. In the stained films the marrow was exceedingly cellular. In most areas there were virtually no cells other than abnormal plasma cells (fig. 1, C). These varied from the size of the ordinary plasma cells to some two or three times as large. Many had double or triple nuclei. The latter contained one, two, or even three prominent nucleoli, some as large as one half the diameter of the nucleus.

The patient was admitted to the hospital on 2/18/48 for a trial of urethane therapy. The drug was tolerated well in divided doses by mouth and between 2/21/48 and 3/9/48, 85 Gm. were given. A leukocytosis of 19,000 developed on the fourth day of therapy. The white blood count then gradually fell to 2,900 on the sixteenth day. Urethane was withheld for seventeen days after which it was resumed in a dose of 15 to 2 Gm. per day until another 35 Gm. had been administered. During the first two weeks in the hospital her temperature ranged from 37 C. to 38.3 C. with one rise to 39.6 C. Afterwards her temperature was normal. Bone pain gradually became less until she was entirely comfortable while resting in bed. After one month in the hospital she was able to return home. There she gradually extended her activity until she could sit at the table with her family for meals and walk by holding to furniture. Pain of sciatic radiation developed after a period of over exertion but this subsided when her activity was again restricted. Three and one-half months after the beginning of therapy she was able to walk unaided and without difficulty.

The peripheral blood values (table 1) showed progressive improvement following the administration of urethane. Immature granulocytes, plasma cells and ocellated red cells disappeared in a few weeks from the circulating blood. After two months of therapy the bone marrow was re-examined. An exceedingly cellular marrow was again obtained but the predominant cells were now normal erythroid and myeloid elements (table 1). Scattered through the films there was an occasional small cluster of plasma cells greatly altered in morphology (fig. 1 E). Their cytoplasm was now irregular in contour, stained a denser blue and often contained light areas suggestive of early vacuolization. Their nuclei were extremely eccentric and pyknotic. Basophilic granules in the cytoplasm such as occur following stilbamidine and antimony therapy were not present. A few of the plasma cells had developed into giant forms nearly as large as megakaryocytes (fig. 1 D). The total number of plasma cells comprised but 1.0 per cent of the total marrow cells. Megakaryocytes, many showing platelet formation, were present in about normal numbers. Other marrow elements were not detectably abnormal.

On a check-up examination three and one-half months after the beginning of treatment the plasma proteins had fallen to 8.1 Gm. per 100 cc., with 5.0 Gm. albumin and 3.1 Gm. of globulin (table 5). The bromsulphalein test repeated as before showed but a trace of the dye remaining in the serum at 45 minutes. The alkaline phosphatase was 6.7 Bodansky units, calcium 10.6 mg. per 100 cc., and phosphorus 2.9 mg. Bence Jones proteinuria and albuminuria were not present.

Improvement in her general health continued during the following five months. The peripheral blood values became normal (table 1). Repeated bone marrow examinations failed to show abnormal numbers or types of plasma cells. The blood chemical values remained unchanged. Skeletal x-rays showed gradual recalcification of the vertebrae, pelvis and upper femora. She began to complain of lower abdominal pain radiating into her right thigh and examinations showed progressive anterior displacement of a fibroid uterus. A laparotomy was performed and the iliac vessels and mesocolon were infiltrated with a fleshy retroperitoneal tumor. Resection was attempted. She died two days later of uncontrolled hemorrhage one month after the beginning of urethane therapy. Pathologic examination of the resected tissue showed it to be a myeloma tumor.

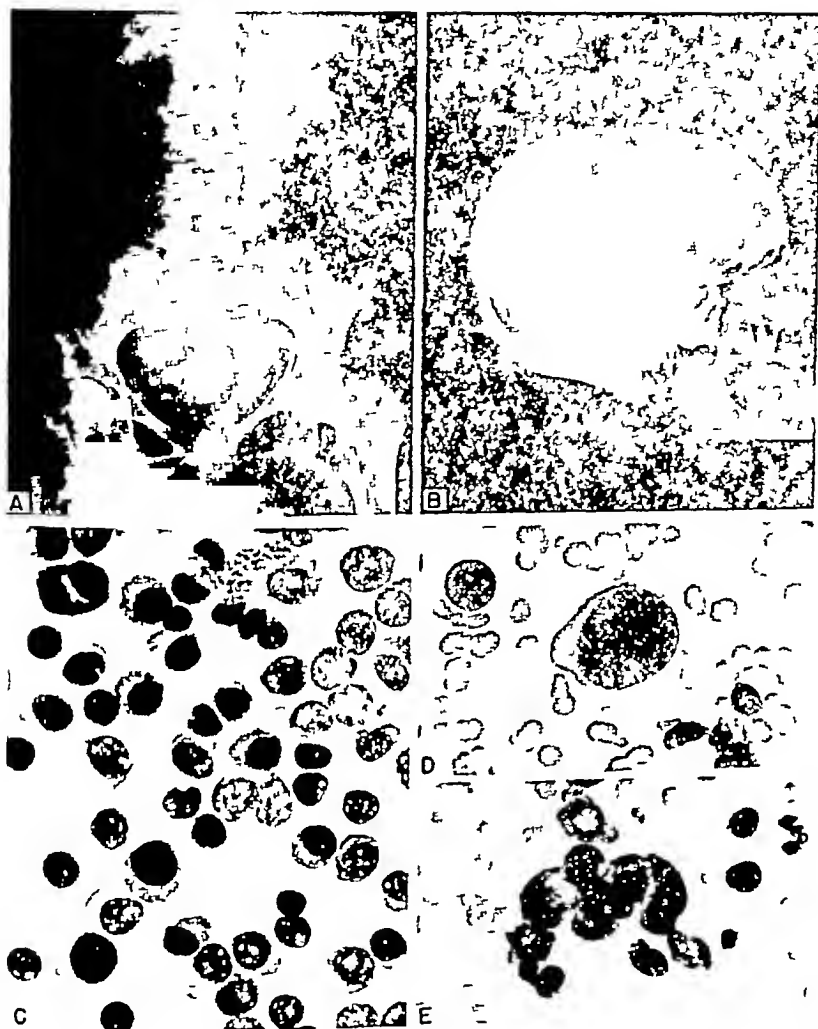


FIG. 1. CASE 1. C, F, H. UNIT No. B-25150

A & B Roentgenograms showing extensive bony destruction in ribs, vertebrae, pelvis, upper ends of femurs, and skull before treatment

C Photograph of aspirated sternal marrow before treatment (X 600) showing preponderance of abnormal plasma cells

D Monstrous abnormal plasma cell (X 600) in aspirated sternal marrow 4 months after beginning of urethane therapy

E Clumped plasma cells with dense cytoplasm and pyknotic nuclei in same specimen as D (X 600)

*Case 2* A R. Unit No. C-19679. This 47 year old white divorced mill worker was referred to Duke Hospital by Dr. A. J. Tannenbaum of Greensboro on October 13, 1947. Her general health had been good until about two years previously when following a marital rift she developed malaise, loss of appetite, and became habitually worrisome. Cramps and pains about her muscles and joints developed during the

following months. In March, 1946, she found that her weight was ten pounds below her average. An anemia with blood values about 50 per cent of normal was discovered and it was thought that she improved for a time with vitamin and liver therapy. Two or three months before her hospital admission, she became more or less constantly uncomfortable due to sharp stabbing pains particularly about her shoulders and trunk. Her family physician after admitting her to a local hospital found that she had fever, anemia, albuminuria and skeletal decalcification. Two blood transfusions were given. Multiple myeloma was considered as a diagnosis but since Bence Jones protein could not be found in the urine a tentative diagnosis of hyperparathyroidism was made.

TABLE 2.—Case 2, A. R., C-19679, Hematologic Findings

Date 1947-48	Hgb	RBC	WBC	Hemato- crit	Reticulo- cytes	Plate- lets	Differential WBC and therapy
	Gm per 100 cc	mill per c mm			%	thou sands per c mm	
10/14	9.1	3.79	10500	34	0.8	300	Neutrophils 70, lymphocytes 17 monocytes 13
	Bone Marrow Plasma cells 35, neutrophils 16, stabs 16, metamyelocytes 14, myelocytes 5, promyelocytes 1, erythroblasts 2, normoblasts 11						
10/20							Urethane 4 Gm /day  (240 Gm.)
10/23	8.9	3.4	6150	29	0.5		
10/25	9.0		15900				
10/28	8.2		7550				
11/24	9.0	3.2	4050	29	1.1		
12/22	10.4	3.7	7300	32	1.3	350	
1/19	11.4	4.0	9350	36	2.3	1280	
	Bone Marrow Plasma cells 7, neutrophils 16, stabs 16, metamyelocytes 8, myelocytes 6, promyelocytes 3, lymphocytes 9, eosinophils 2, reticulum cells 3, erythroblasts 1, normoblasts 29.						
3/15	10.9	3.9	11050	36	1.0	671	Neutrophils 72, stabs 3, lymphocytes 6, monocytes 14, eosinophils 5
5/10	11.2	3.9	10850	36	2.7	310	
	Bone Marrow Plasma cells 4, neutrophils 17, stabs 22, metamyelocytes 12, myelocytes 3, myeloblasts 3, lymphocytes 5, eosinophils 2, reticulum cells 4, basophilic normoblasts 5, acidophilic normoblasts 12, erythroblasts 1						
6/21	12.9	4.4	10600	40	1.0	1900	

Physical examination showed the patient to be a poorly nourished, chronically ill middle aged white woman. Pressure over the lower ribs was painful. The liver edge was palpable just below the costal margin. There were no enlarged lymph nodes or tumor masses.

Examination of the peripheral blood showed a normochromic, normocytic anemia (table 2). There was marked rouleau formation in the blood films. There was a large amount of protein in the urine and on some occasions a small amount of Bence Jones protein could be demonstrated. Serologic tests for syphilis were negative. The serum proteins were 8 Gm per 100 cc, with albumin 2.5 Gm and globulin 5.5 Gm. The blood calcium was 9.1 mg per 100 cc, phosphorus 3.8 Gm and the alkaline phosphatase 1.7 Bodansky units. A bromsulphalein test of liver function using 5 mg of the dye per kilogram of body weight showed 10 per cent retention after 45 minutes. A phenolsulphthalein test of renal function

showed an excretion of 40 per cent of the dye in two hours. Roentgen examination of the skeleton (fig. 2, A) showed generalized, punched-out destructive lesions in the skull, ribs, scapulae, spine and pelvis. There were questionable areas of bony destruction in the upper end of the right tibia. There was no collapse of the vertebrae. Bone marrow was obtained by aspiration from the sternum and from the spinous process of a lumbar vertebra. Thirty-five per cent of the marrow cells were immature and abnormal plasma cells (fig. 2, C).

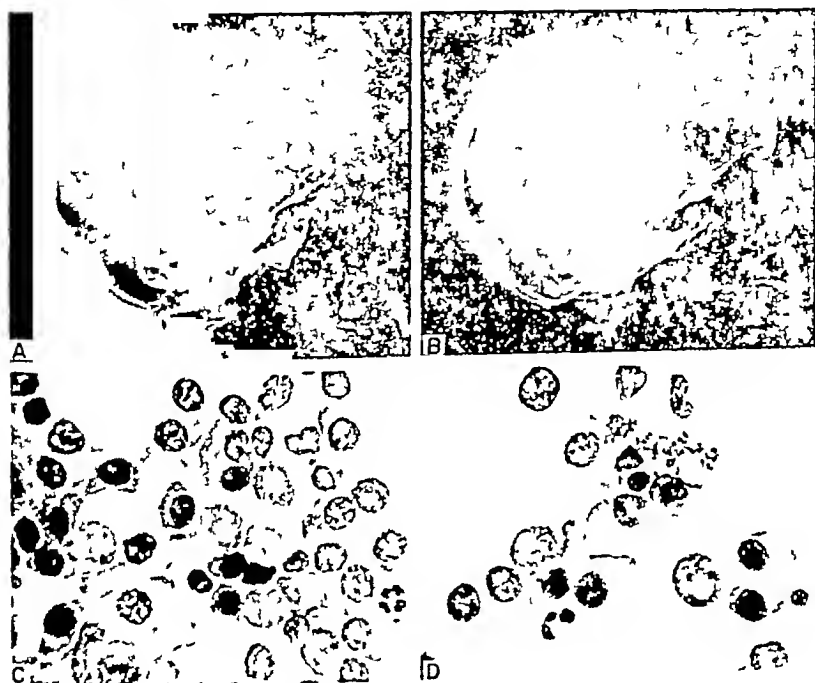


FIG. 2. CASE 2, A. R. UNIT No. C 19679

- A Roentgenograms of skull showing multiple small areas of bony destruction before treatment
- B Repeat roentgenogram seven months after beginning of treatment showing no progression in lesions
- C Photograph of aspirated bone marrow ( $\times 600$ ) before treatment showing 35% atypical plasma cells
- D Photograph of clumped plasma cells in aspirated marrow three months after beginning of urethane therapy. Plasma cells were reduced to 4% with variation in size and altered staining reaction

The administration of urethane was started on October 20, 1947, giving 4 Gm. per day by mouth. She tolerated it well. On the fourth day of therapy a leukocytosis of 15,900 occurred but during the next month the WBC gradually fell to hover around 4,000. During her two weeks in the hospital she had a daily rise in temperature to 38–40  $^{\circ}$ C. At the end of this time bone pain was decreasing, she had begun to gain weight and was able to return home. She was seen thereafter at frequent intervals for check-up examinations. After a few more weeks her temperature remained normal. Her blood values improved progressively (table 2). The urethane was discontinued after two months of continuous therapy with a total dose of 240 Gm. had been given. Re-examination of the bone marrow three months after the beginning of therapy showed that the plasma cells were reduced to 4 per cent. The remaining marrow elements were not notably abnormal.

Seven months after the start of treatment she had gained 20 pounds in weight. She considered her general health better than it had been for several years. She was able to do all of her work at home except the heaviest tasks. Physical examination at this time showed no abnormalities. Bence-Jones protein could not be demonstrated in the urine. The serum proteins were 8.8 Gm. per 100 cc. as before but the albumin was now 4.4 Gm. and the globulin 3.6 Gm. (table 5). Repeat x-ray films of the skull showed that there had been no progression in the bony lesions. Multiple areas of rarefaction remained (fig. 2, B).

Bone marrow was aspirated from the sternum for the third time. The cellularity was within range of normal with a total white cell count of 232,000. Four per cent of the cells were plasma cells. These varied in size to an unusual degree from about 10 to 50 microns in diameter (fig. 2, D). Their cytoplasm was somewhat indefinite as to boundary and their staining reaction varied from dark to pale blue from cell to

TABLE 3—Case 3, S J C C-28643 Hematologic Findings

Date 1948	Hgb	RBC	WBC	Hemato- crit	Reticulo- cytes	Plate- lets	Differential WBC and therapy
	Gm. per 100 cc.	mill. per c mm.			%	thous. ands per c mm.	
2/24	7.2	2.3	5000	21	4.0	214	Neutrophils 60, stabs 4, metamyelo- cytes 2, myelocytes 2, promyelo- cytes 2, lymphocytes 26, monocytes 4, normoblasts 4/100 WBC.
	Bone Marrow Plasma cells 68, neutrophils 20, stabs 2, metamyelocytes 1, eosinophils 1, basophils 2, lymphocytes 3, monocytes 1, normoblasts 2.						
3/3							Urethane 4 Gm./day
3/4	6.9	2.2	4450	20	3.4	880	
3/9	6.4	2.5	5200	21	3.2	925	
3/15	7.5	2.5	4350	23	6.9	204	
3/22	8.6	2.9	3150	24	0.4	150	
3/26	8.2	2.9	2900	24	4.6	75	Urethane 2 Gm./day (268 Gm.) Neutrophils 72, stabs 4, lympho- cytes 14, eosinophils 2, monocytes 8
4/5	8.5	3.0	3650	27	3.0	1000	
4/26	9.0	2.7	3000	25	5.0	590	
5/10	9.5	3.0	3600	27	7.3	306	
5/17	9.9	3.0	2350	27	5.1	750	
5/28	10.8	3.46	3900	31	3.1	858	
6/18	10.0	3.3	3400	31	2.3	343	

cell. Basophilic granulation was not present. The nuclear chromatin stained densely and occurred in coarse clumps. The appearance and distribution of the remaining marrow elements was not abnormal.

A check up examination at eight months showed no essential changes. She had returned to full time work in a hosiery mill. The blood values had continued to improve (table 2). The urine contained a trace of Bence-Jones protein. A phenolsulphonthalein test showed an excretion of 35 per cent of the dye in two hours. The alkaline phosphatase activity had become slightly elevated.

A few weeks later she felt feverish for a day or two. Examination showed more Bence-Jones protein in the urine and an increased number of plasma cells in the bone marrow. She was given 120 Gm. of urethane in a month's time. On a check up examination thirteen months after urethane therapy was first begun she was working full time and there was no evidence of further relapse.

Case 3, S J C Unit No C-28643. This 54 year old electrician was admitted to Duke Hospital on February 23, 1948, for the investigation of anemia, suspected heart and renal disease. He had been well and working regularly until four weeks previously when within a period of a few days he became weak, pale and unable to exert himself physically without becoming short of breath. He had pain about the lower

ribs on the right side that was made worse by breathing and coughing. His symptoms became progressively worse. During the week preceding his hospital admission he was quite drowsy in the afternoons, had little appetite, was often nauseated and vomited once or twice. He found that he slept better at night when using two or three pillows. Early in the morning of the day of his hospital admission he was awakened from sleep by acute dyspnea.

Physical examination disclosed a pale, overweight white male, obviously ill. His blood pressure was 135/80 mm. of mercury. Two small flame shaped hemorrhages were visible near the right optic disc. His

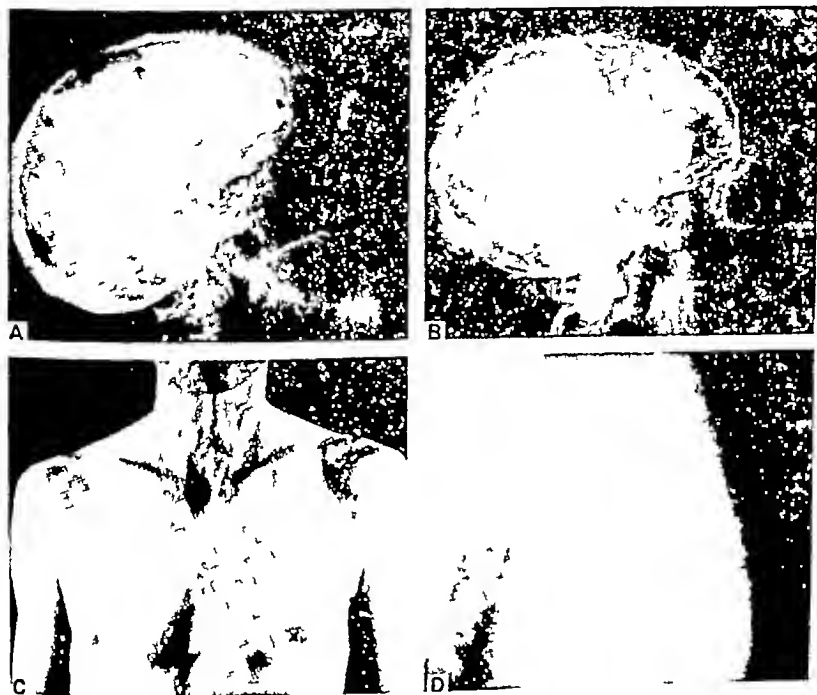


FIG. 3. CASES 3 AND 4

A. Case 3, S. J. C. Unit No. C-28643. Roentgenogram of skull before treatment showing multiple large and small areas of bony destruction.

B. Case 4, O. M. R. Unit No. C-17783. Roentgenograms of skull showing similar but less extensive bony destruction.

C and D. Photograph and lateral roentgenogram of myeloma cell tumor of sternum, case 4.

heart was slightly enlarged. There was a moderately loud systolic murmur at the apex and the first heart sound was doubled. The liver edge was felt 5 cm. below the right costal margin. The tip of the spleen was palpable. There was slight pitting edema at the ankles.

Laboratory studies showed that he had a severe normochromic, normocytic anemia with immature granulocytes and nucleated red blood cells in the circulating blood (table 3). In fresh and stained films there was conspicuous rouleaux formation. Serologic tests for syphilis were negative. Several urinalyses showed heavy proteinuria and granular casts but no Bence Jones protein. Blood chemical determinations showed the NPN to be 35 mg. per 100 cc., total proteins 8.5 mg. with albumin 3.8 Gm. per 100 cc. A phenolsulfonphthalein test showed impaired renal function: 5 per cent of the dye appearing in the urine in fifteen minutes and a total of 40 per cent in two hours. The bromsulfalein test of liver function, using 5 mg. of the dye per kilogram of body weight, showed only a trace of the dye appearing in the



tumor in October 1947. During the next few months he had far less pain about the sternum but the tumor mass did not decrease in size.

He was recalled for a check up examination on April 12, 1948. At that time he complained of more pain than on previous occasions, especially when he exerted himself physically. The discomfort was noted particularly over the left upper humerus, about his ribs and sternum. Physical examination showed no obvious changes. Examination of the blood showed that there had been some deterioration in the blood values (table 4). A specimen of bone marrow from a spinous process was again examined and 39 per cent of the cells were abnormal plasma cells. The serum proteins had risen to 10.1 Gm. per 100 cc. with albumin 2.9 Gm. and globulin 7.2 Gm. (table 5). X-ray films of the chest, skull, left humerus and sternum showed little definite extension of the areas of previous bone destruction.

The administration of urethane was started on April 13, 1948, with a dose of 4 Gm. per day by mouth. During the next four weeks his pain became gradually less except about an area on the lateral chest wall where a rib had apparently been fractured during the maneuvers incident to making roentgen films. Examination of the blood showed that a leukopenia had developed. An increased number of plasma cells was still present in the bone marrow but they were now larger in size and their cytoplasm more densely stained. Their nuclei were extremely eccentric and the chromatin was aggregated into dense clumps.

The administration of urethane was continued at home under the supervision of his family physician. The leukopenia persisted but became no more severe. Without notable trauma another rib fracture occurred and severe pain developed about one hip. He was admitted to his local hospital and the pain subsided at bed rest.

The urethane was used somewhat irregularly. On a check up examination three months after the beginning of treatment he considered himself definitely improved. Urethane was discontinued and for two months he was virtually free of symptoms. Skeletal pain then gradually reappeared and he did not return for further treatment. He died at home seven months after the beginning of urethane therapy.

## DISCUSSION

Multiple myeloma is a malignant disease resulting from the excessive proliferation of abnormal plasma cells within the bone marrow. The clinical course is variable, as judged by the length of survival of individual patients, but uniformly progressive and ultimately fatal. There is no evidence that real spontaneous remissions occur.

Therapy in the past has been unsatisfactory. The commoner agents useful in the treatment of the lymphomatous diseases, roentgen irradiation, radioactive phosphorus, and nitrogen mustard compounds, have little demonstrable effect beyond relieving pain in some individuals. Stilbamidine, pentamidine, and antimony compounds have been reported to relieve skeletal pain and produce apparently specific granular changes in the cytoplasm of the myeloma cells.

The 4 patients included in this study illustrate many of the variable clinical features of multiple myeloma. In Cases 1 and 3 the disease was rapidly progressive. In the other two it was progressing slowly. Case 4 was thought at first to have a solitary myeloma cell tumor of the sternum. Examination of marrow obtained from other bones showed the disease to be generalized. The major effect of roentgen therapy to the sternal tumor was relief of pain. In Case 1 the predominant feature was a devastating skeletal disease. Metastatic carcinoma was considered a good possibility, as it frequently must be, in the differential diagnosis. In Case 3 symptoms of cardiac failure were precipitated by anemia. The presence of renal disease complicated the diagnostic problem. The finding that the anemia did not result from renal failure, however, and the occurrence of immature granulocytes in the circulating blood led to a bone marrow examination and the discovery of plasma

cell overgrowth. There was no skeletal pain and roentgen films showed significant abnormalities only in the skull. In all patients the crucial diagnostic information was provided by examination of the bone marrow.

The treatment adopted in these cases was entirely empiric. The urethane was given in divided doses by mouth in the form of an elixir or syrup. All four of the patients tolerated the chemical exceptionally well with virtually no gastrointestinal symptoms. During the first three to four weeks they were given 4-6 Gm. per day. Three patients developed leukopenia following which the dose was reduced to about 2 Gm. daily or temporarily suspended. Further fall in the white cell count did not occur. The urethane was given over a period of about two months and then discontinued. The total dose per patient varied from 120-240 Gm. Treatment and post-treatment periods of observation ranged from seven to thirteen months. Two patients relapsed and were given a second course of therapy.

All 4 patients showed much the same type of response to urethane. General clinical improvement appeared during the second and third week of therapy when skeletal pain and fever began to subside. Physical activity soon became tolerable within the obvious limitations imposed by skeletal disease. The two patients with most extensive areas of skeletal destruction were able to perform ordinary activities and do light work within four to six months of the start of treatment without discomfort.

A normochromic, normocytic anemia with hemoglobin values ranging from 7.2 to 11.0 Gm. was present in all 4 patients. In 2 cases, immature granulocytes and nucleated red cells were present in the circulating blood. An abrupt but transient leukocytosis was noted in 2 patients on the fourth day of urethane administration, possibly the result of chemical stimulation of cell division. Leukopenia with white cell counts ranging between 2600 and 4400 developed in about three to four weeks. Reduction in the urethane dosage to around 2 Gm. per day was sufficient to prevent the development of more serious white cell depression. In about the same time evidence of marrow crowding subsided, immature granulocytes and nucleated red blood cells disappearing from the circulating blood. Progressive fall in hemoglobin and red cell values ceased after one to two weeks of urethane but notable regeneration of blood did not begin before four to six weeks. There was gradual improvement in the blood values toward normal for several weeks following the termination of urethane therapy.

The initial bone marrow aspiration showed in all cases the massive proliferation of abnormal plasma or myeloma cells diagnostic of multiple myeloma. Repeated examinations of bone marrow in Cases 1, 2, and 3 obtained from different sites after urethane therapy revealed a striking quantitative decrease in the number of these cells. A characteristic change in myeloma cell morphology occurred, also, with urethane administration. Some became monstrous in size recalling the changes observed in plant seedlings.<sup>1</sup> Others as observed in spread films made from aspirated marrow tended to adhere together in small compact clumps. Variation in cell size, densely staining cytoplasm, and eccentric and pyknotic nuclei were general features of those myeloma cells which persisted after urethane administration. Basophilic granulation of the cytoplasm<sup>2-4</sup> did not develop.

The initial blood chemical values (table 5) were typical of those occurring in multiple myeloma. In Case 1, the serum calcium was elevated to 12.4 mg per 100 cc, but in all other instances it was normal. Phosphatase activity, determined in 3 cases before treatment, was low or normal and in 2 patients with extensive skeletal disease it increased slightly after urethane administration.

Hyperglobulinemia with reversal of the albumin globulin ratio was present initially in all cases. In Cases 1, 2, and 3 the serum globulin fell markedly and the albumin rose slightly to restore a normal ratio of these protein components during the period of after treatment follow-up. The serum proteins were studied electrophoretically in these 3 cases before and after urethane treatment, and will be reported in detail later by Dillon and Rundles.<sup>28</sup> In Case 1, 49 per cent of the total protein showed the electrophoretic mobility of gamma globulin. Four months after the beginning of urethane therapy protein with this mobility was reduced to 18.0 per cent. In Case 2, a similar increase in the gamma globulin occurred, amounting to 45.7 per cent of the total. Seven months after urethane administration was begun this component was reduced to 23.8 per cent. In Case 3, the initial electrophoretic study showed that the abnormal protein had a boundary lying between the beta and gamma globulins, the M variety of protein abnormality in multiple myeloma described by Gutman, et al.<sup>29-30</sup> The total percentage of M and gamma globulins before treatment totaled 45.2 per cent. Three and one-half months later these fractions had fallen to 33.4 per cent.

Renal disease<sup>31-32</sup> as evidenced by proteinuria of greater or less degree was present in all of the patients with multiple myeloma. Bence-Jones protein was demonstrated in the urine of two. None had nitrogen retention. The excretion of phenol-sulphonphthalein dye was impaired in two patients before treatment was started. During the weeks following urethane administration nitrogen retention did not develop, albuminuria tended to subside and Bence-Jones proteinuria was less often demonstrable.

Serial roentgen films taken over a period of months following urethane therapy showed no progression in the destructive skeletal lesions which are so prominent a feature of multiple myeloma. The subsidence of bone pain occurring at bed rest, followed in a few weeks by the ability to tolerate moderate physical activity, suggested improvement in skeletal support as a result of therapy. The slight increase in phosphatase activity suggests some attempt at bony repair. There was definite roentgen evidence of recalcification in one patient 6 months after the beginning of treatment. Bony softening persists without doubt for some period of time, and perhaps indefinitely. Roentgen films of the skeleton thus provide no means for judging the early response to therapy, but the chronicity of the lesions does caution against the too rapid expansion of physical activity.

Evidence has been presented to show that the administration of urethane to patients with multiple myeloma alters the fundamental abnormalities of the disease in a selective and beneficial way not possible by previously available therapeutic agents. The present report concerns only the early responses to treatment. The long term results remain to be studied. At this time we have no doubt that urethane therapy has already prolonged life in 2 cases in whom the disease was

rapidly progressive Relapses of the disease may not occur for as long as six months or longer after discontinuation of therapy Whether interrupted or continuous therapy will eventually prove most desirable is a matter of conjecture To detect reactivation of the disease so that therapy can be given again when indicated will require serial blood examinations, frequently repeated bone marrow studies with attention to the number and appearance of the plasma cells, quantitative study of proteinuria, and serial studies of the serum proteins preferably by electrophoretic methods The effect of urethane therapy offers a new tool in investigating the complex protein and cellular abnormalities which characterize the disease

### CONCLUSIONS

Four patients with multiple myeloma have been treated with urethane (ethyl carbamate) for eight to ten weeks in total doses of 120-290 Gm and observed over periods ranging from seven to thirteen months Striking benefit relating to all aspects of the disease was observed Fever, skeletal pain and acute symptoms subsided after two to four weeks of therapy In individuals with severe anemia, immature granulocytes and nucleated red cells disappeared from the circulating blood, and over a period of several weeks, the blood values improved greatly toward normal Abnormal plasma or myeloma cells decreased quantitatively in the bone marrow and underwent morphologic changes indicative of retarded or arrested growth The serum protein abnormalities characteristic of multiple myeloma became less pronounced or disappeared as did the albuminuria and Bence-Jones proteinuria Serial roentgenograms of the skeleton showed no progression in the destructive lesions There was little evidence of skeletal recalcification, however, for four to six months after treatment The long term results of urethane therapy in multiple myeloma, the liability to exacerbation of the disease, the effectiveness of subsequent courses of urethane therapy, the course of the associated renal disease, the extent of skeletal recalcification and repair, etc., are matters for further study

### REFERENCES

- <sup>1</sup> TEMPLEMAN, W A AND SEXTON W A Effect of some arylcarbamic esters and related compounds upon cereals and other plant species *Nature* 156 630 1945
- <sup>2</sup> HADDOW, A AND SEXTON W A Influence of carbamic esters (urethanes) on experimental animal tumors *Nature* 157 500 1946
- <sup>3</sup> PATERSON E, AP-THOMAS I, HADDOW, A AND WAYKINSON J M Leukemia treated with urethane compared with deep x ray therapy *Lancet* 1 677 1946
- <sup>4</sup> HIRSCHBOECK, JOHN S, LINDERT M C F, CHASE JULES AND CALVIN THOMAS L Effects of urethane in the treatment of leukemia and metastatic malignant tumors *J A M A* 136 90 1948
- <sup>5</sup> HUGGINS, C, YU S T AND JONES R Inhibitory effects of ethyl carbamate on prostatic cancer *Science* 106 147 1947
- <sup>6</sup> LEUCUTIA, T Urethane therapy *Am J Roentgenology* 59 421 1948
- <sup>7</sup> MARINOER S Urethan-Behandlung bei Leukämia *Schweiz Med Wschr* 77 124 1947
- <sup>8</sup> BEDINOER PAUL L, PAUCHER HENRY G AND LIMARZI LOUIS R Effect of urethane on leukemia *J Lab & Clin Med* 32 1394 1947
- <sup>9</sup> BERMAN LAWRENCE AND AXELROD ARNOLD R Effect of urethane on malignant disease *Am J Clin Path* 18 104 1948
- <sup>10</sup> DUSTIN P Ethyl carbamate in treatment of leukemia *Bruxells Medical Bulletin* 75 1-2 1948
- <sup>11</sup> GOODMAN M J AND LEWIS H P Urethane in leukemia *J A M A* 137 1175 1948

- <sup>12</sup> SCHULZE, E Die Behandlung Chronischer Leukamien mit Urethan Deutsche Med Wchnschr 72 153 1947
- <sup>13</sup> WEBSTER J J Urethane in leukemia J A M A 133 901 1947
- <sup>14</sup> CRAVER, LLOYD F Recent advances in treatment of lymphomas, leukemias and allied disorders Bull N Y Acad Med 24 3 1948
- <sup>15</sup> BAYRD EDWIN D AND HECK, FRANK J Multiple myeloma. J A. M. A 133 147 1947
- <sup>16</sup> LICHTENSTEIN, LOUIS AND JAFFE H L Multiple myeloma Arch Path 44 207, 1947
- <sup>17</sup> GARLAND L HENRY AND BAYLES, R. KENNEDY Roentgen treatment of multiple myeloma Radiology 50 297 1948
- <sup>18</sup> GOOTNICK L. T Solitary myeloma Radiology 45 385 1945
- <sup>19</sup> REINHARD EDWARD A, MOORE CARL V, BIERBAUM OLGA S, AND MOORE, SHERWOOD Radioactive phosphorus as a therapeutic agent J Lab & Clin Med 31 107 1946
- <sup>20</sup> RHODAS C P Nitrogen mustards in the treatment of neoplastic disease J A M A 131 656 1946
- <sup>21</sup> SPURR CHAS L. SMITH TAYLOR R., AND JACOBSON, LEON O Chemotherapy in human lymphomas leukemias, and allied disorders of the hemopoietic system. Radiology 50 387, 1948
- <sup>22</sup> WINTROBE M M HUGUELY CHAS M, McLENNAN M. T AND LUCIO PENNA DE CARVALHO LIMA Nitrogen mustard as a therapeutic agent for Hodgkin's disease, lymphosarcoma and leukemia Ann Int Med 27 529 1947
- <sup>23</sup> JACOBSON, LEON O, SPURR, CHARLES L. GUZMAN BARRON E. S, SMITH TAYLOR R, LUSHBAUGH CLARENCE AND DICK, GEORGE F Nitrogen mustard therapy J A M. A 132 263 1946
- <sup>24</sup> SNAPPER, ISIDORE Stilbamidine and pentamidine in multiple myeloma. J A M. A. 133 157 1947
- <sup>25</sup> ——— MIRSKY A E RIS H SCHNEID B AND ROSENTHAL, M. Development of inclusion bodies containing ribose nucleic acid in myeloma cells after injections of stilbamidine Determination of stilbamidine in myeloma tissue Blood 2 311 1947
- <sup>26</sup> RUBINSTEIN M. A Chemical treatment of myeloma Use of antimony Preliminary report. Blood 2 555 1947
- <sup>27</sup> ALWALL, N Urethane and stilbamidine in multiple myeloma Lancet 233 388, 1947
- <sup>28</sup> DILLON M L AND RUNDLES R. W (to be published)
- <sup>29</sup> GUTMAN, A B MOORE D H, GUTMAN E. B McCLELLAN, VIRGINIA AND KABAT E. A Fractionation of serum proteins in hyperproteinemia with special reference to multiple myeloma J Clin Investigation 20 765 1941
- <sup>30</sup> MOORE DAN H KABAT E A AND GUTMAN, A B Bence Jones proteinuria in multiple myeloma J Clin Investigation 22 67 1943
- <sup>31</sup> FURBUS, W D PERLZWEIN W A PARFENTJEV I A AND BURWELL, J C Bence Jones protein excretion and its effect upon the kidney Bull Johns Hopkins Hosp 57 47 1935
- <sup>32</sup> BLACKMAN SAM S BARKER W H BUELL, MARY V AND DAVIS B D On the pathogenesis of renal failure associated with multiple myeloma Electrophoretic and chemical analysis of protein in urine and blood serum J Clin Investigation 23 163 1944

# THE RESPONSE OF EOSINOPHILS IN THE GUINEA PIG TO SENSITIZATION, ANAPHYLAXIS AND VARIOUS DRUGS

By MAX SAMTER, M D

## I REVIEW OF EARLY CONCEPTS AND EXPERIMENTAL STUDIES SCOPE

THE HISTORY of the eosinophilic polymorphonuclear leukocytes (referred to as eosinophils throughout this study) has few landmarks. In 1846, Wharton Jones<sup>1</sup> gave the first reliable description of 'coarse granules' in colorless blood cells. Their morphologic characteristics are so striking that early (Max Schultze<sup>2</sup>) and recent (Cunningham and Tompkins<sup>3</sup>) observations differ only in insignificant detail. Paul Ehrlich<sup>4</sup> established in 1879—in his *farbenanalytischen Untersuchungen*—the distinctive mark of the fixed cell, namely, the elective staining of its  $\alpha$ -granules with acid dyes.

Since then, innumerable data on the occurrence of eosinophils have been collected. A comprehensive survey prepared by Emil Schwarz<sup>5</sup> in 1914 listed in its 653 pages a bibliography of 2758 publications. Yet while Schwarz and others defined the occurrence of eosinophils under various clinical and experimental conditions, they added little to our knowledge of their function. The association of eosinophilia with a variety of unrelated disorders is still not well understood (Bethell, Sturgis, Rundles and Meyers<sup>6</sup>).

Experimental research has been retarded for two reasons. The first reason is the direct result of the controversy about the site of origin of the eosinophils. Authors who regard the bone marrow as their only source, will interpret circulating eosinophils as the necessary link between their site of formation and the tissues in which they are eventually found. Authors who accept the concept of the development of eosinophils at sites other than the bone marrow, have concluded that the same circulating eosinophils represent an overflow, or have been discarded from the tissues in which they have developed.

Ehrlich<sup>7</sup> committed himself to the hypothesis that eosinophils are formed in the bone marrow and are distributed by the blood stream. His concept initiated a lively scientific controversy about the question of the homoplastic or heteroplastic origin of eosinophils which fills the early volumes of *Folia Haematologica* (Ascoli<sup>8</sup>, Pappenheim<sup>9</sup>, Weidenreich<sup>10</sup>, Maximow<sup>11</sup>, Downey<sup>12</sup> and Ringhorn<sup>13</sup>).

Biggart<sup>14</sup> has summarized some of the controversial questions: whether or not blood and tissue eosinophils are identical; whether there is local multiplication of eosinophils; assuming that there is only one type of eosinophil, what causes their emigration from the blood into the tissues; what finally are the relations between bone marrow and tissue eosinophilia?

Several of these questions have been studied by Opie<sup>15</sup>, Schlecht and Schwenker<sup>16</sup>, Weinberg and Seguin<sup>17</sup> and Homma<sup>18</sup>. The majority of investigators have recognized the bone marrow as the source of the eosinophils which appear in the peripheral circulation, but a final agreement between the dissenting factions has not been reached. Cooke<sup>19</sup> has only recently claimed that the discrepancy between the number of eosinophils in blood and tissue are sufficient reason to assume the existence of two distinct

From the Department of Medicine and the Allergy Unit, College of Medicine, University of Illinois at Chicago, Ill.

The expenses of this investigation were defrayed in part by funds from the Allergy Club, Chicago, Ill.

types of eosinophils. And while Homma<sup>18</sup> emphasizes the fact that no intermediary stages exist between small lymphocytes, large lymphocytes and macrophages on one side eosinophils on the other side Ringoe<sup>20</sup> concludes "Tissue eosinophils are derived from various sources, lymphocytes large mononuclears, plasma cells and adventitial cells being regarded as parent cells".

In spite of the existing disagreement as to the site of origin of eosinophils, the intimate relationship of eosinophilia to certain phenomena of hypersensitivity has been established beyond doubt (Hajos,<sup>21</sup> Hajos and Mazgon,<sup>22</sup> Campbell, Drennan and Rettie,<sup>23</sup> and D. H. Campbell<sup>24</sup>). Its mechanism, however, as well as its function and meaning are still obscure. It is surprising to note that experiments of very similar nature have led investigators to diametrically opposed conclusions. Analysis of the literature reveals what must be regarded as the second reason for the hesitant progress of experimental research on eosinophils: a multitude of experimental procedures in a field where minimal changes in technic are bound to cause major changes in results.

It will become necessary, therefore, to apply the rigid standards which distinguish immunologic research in general to research on eosinophils which are part of the immuno-reactions of the organism. Study of eosinophilic response requires a uniform experimental technic which includes a uniform species, a uniform antigen, and a uniform route of administration. Accordingly, the first section of this investigation discusses criteria for the choice of the experimental animal, the choice of a suitable antigen, its dose, and the route by which it is administered.

## II. GENERAL CONSIDERATIONS

### *Choice of the Experimental Animal*

Review of the literature shows that guinea pigs have been preferred for study of experimental eosinophilia although other species have been investigated. Data about hematologic changes in rabbits and dogs, however, are scanty and controversial. White rats—used by Homma<sup>18</sup> in an attempt to correlate eosinophils in bone marrow, blood and tissues after injection of parasites and parasitic material—have the disadvantage that they are very unresponsive to anaphylactic sensitization. If one intends to demonstrate the allergic state of the sensitized animal by the symptoms of anaphylaxis, guinea pigs are most suitable and were therefore employed.

The guinea pigs were bought at random and raised until they weighed between 400 and 450 grams at the time of the experiment. It appeared advisable to eliminate larger guinea pigs, since Opie<sup>15</sup> in his early studies had shown that guinea pigs tend to develop a spontaneous eosinophilia which increases with increasing weight. The cause of this eosinophilia has not been established although parasitic infestation such as by *megastoma entericum* in the small intestines and *infusoria* in the cecum has been suspected. The diet consisted of oats supplemented by lettuce and carrots, no added water was given. The average differential count prior to sensitization of the 482 animals used in our studies was as follows: Lymphocytes 42 per cent, neutrophils 55 per cent, monocytes 3 per cent. Eighty-two per cent of the animals had no eosinophils, 14 per cent (68) had 1 per cent. The remaining guinea pigs which had up to 7 per cent eosinophils were classified as a separate group.

They are included and discussed in section VI. The figures at which we have arrived differ slightly from those quoted by other observers, it is felt that this difference may be due, in part, to differences in strains used by this author. The figures listed in Klieneberger's<sup>15</sup> manual, however, are based on too few animals to serve as a satisfactory base line.

Each experimental group consisted of either six or eight animals, male or female, and no sex distinction was made in our studies.

### *Choice of the Antigen*

Selection of a suitable antigen became an interesting problem since Campbell<sup>24</sup> correlated the insolubility of the antigen as well as the presence of -SH groups with its ability to elicit eosinophilia. The literature is full of curious contradictions. Homma<sup>18</sup> reported that coagulated egg albumen and fibrin failed to produce a significant tissue eosinophilia in white rats. Schlecht,<sup>25</sup> on the other hand, was

TABLE I—*Choice of Antigen*

8 guinea pigs sensitized and re-injected with Ovalbumin 20 hours after re-injection	8 guinea pigs sensitized and re-injected with Ovomucin 20 hours after re-injection	8 guinea pigs sensitized and re-injected with Hapamine 20 hours after re-injection
2	8	14
0	2	6
Fatal shock	9	10
5	7	9
8	8	6
Fatal shock	Fatal shock	10
Fatal shock	1	5
4	16	9

Percentage of eosinophils (twenty hours after re-injection) in a consecutive series of guinea pigs sensitized and re-injected intracardially with proteins of strong, moderate and low antigenicity.

able to observe (in two experimental animals) extraordinary increase in eosinophils after injection of a 2 per cent fibrin solution, neither author gives sufficient data about the preparation of the antigen to permit evaluation of the discrepancies. We have conducted a considerable number of preliminary experiments in order to establish variations of the response of eosinophils in guinea pigs to antigens of various solubility and antigenicity as expressed by the severity of anaphylactic reactions following re-injection. The antigens tested included fibrin, several fractions of egg white prepared for us by Dr. A. G. Cole, and hapamine (histamine conjugated with a despeciated horse serum globulin through azo linkage) a compound reported to be a poor antigen (Cohen and Friedman<sup>26</sup>).

Table I summarizes some of the results. The animals had been sensitized twenty-one days before the test by intraperitoneal injection of 75 cc. of a 1 per cent solution of the protein under investigation. They were re-injected intracardially with the homologous antigen. Differential counts were taken of the surviving animals three hours and twenty hours after re-injection. The percentage of eosinophils found after twenty hours (when the maximal eosinophilia had been reached) is



listed in table 1 Guinea pigs sensitized and reinjected with ovalbumin show the lowest percentage of eosinophils, but the highest incidence of fatal reactions, while guinea pigs sensitized and reinjected with hapamine develop a considerable eosinophilia in spite of the absence of fatal shock. The anaphylactic reaction as well as the eosinophilic response are phenomena of sensitization, since neither can be elicited by the introduction of a nonspecific protein, it is evident from our experiments, however, that the effectiveness of a given protein in achieving anaphylactic sensitization does not parallel its ability to produce eosinophilia.

The antigen which was finally selected for this study, horse serum, combined reliable anaphylactic antigenicity with satisfactory ability to produce eosinophilia. With few exceptions, therefore, which are labeled as such, horse serum was used as sensitizing and shocking antigen throughout the experiments. One group of six guinea pigs was sensitized and shocked concurrently with each series of experiments. No conclusions were drawn unless fifty per cent of the controls succumbed to fatal anaphylactic reactions.

The animals which were sensitized by intraperitoneal injection of horse serum with few exceptions did not develop eosinophilia prior to reinjection of the specific antigen. Animals which had more than 1 per cent eosinophils prior to reinjection were excluded from the experiments.

Von Pirquet and Schick<sup>23</sup> had suspected that if a large amount of antigen is injected, a portion of it might persist, unaltered, throughout the period of sensitization, combine with the antibodies which have formed during this period and account thus for the symptoms of serum sickness. Since eosinophils appear in the peripheral circulation subsequent to antigen antibody reactions, it seemed conceivable that the injection of a sizeable dose of horse serum might, similarly, cause eosinophilia. However, in a group of six guinea pigs which were given a sensitizing dose of 20.0 cc. of horse serum, none developed eosinophilia prior to reinjection of the specific antigen.

### *Route of administration*

A survey of experimental research on eosinophils indicates that little attention has been paid to the route by which the antigen was administered. It is a common occurrence to find intradermal, intramuscular, intraperitoneal, intravenous and intracardial administration used indiscriminately within the same group of experiments as if the route of administration were of no consequence. Even if one disregards fundamental objections (Heidelberger, Treffers and Freund,<sup>24</sup>) it is quite obvious that reinjection of antigen into a shock tissue, e.g., the skin, with a resulting local antigen-antibody reaction, creates experimental conditions which cannot be compared with, for example, vascular reinjection. Applied to the study of eosinophils, this variety of routes used for the administration of the antigen makes it understandable why the question has not been adequately answered whether the eosinophilic response to reinjection of antigen is due to the antigen per se, the antigen-antibody reaction, the shock syndrome, or the liberation of substances during the immunologic processes. The experiments of Weinberg and Seguin<sup>17</sup> are a case in point. The results of their observations are significant for

certain phases of the mechanism of the eosinophilic response which will be discussed later, but they also demonstrate clearly that results obtained after subcutaneous, intraperitoneal, and intravenous reinjection are not comparable

Although it is possible to sensitize guinea pigs by any parenteral route, the intraperitoneal injection is not only most convenient but known to give, for this particular antigen, consistent anaphylactic sensitization. The reason for this is not fully understood although it has been suggested that the copious lymphatic drainage of the peritoneum, and a resulting efficient elaboration of antibodies, might be responsible. The sensitizing dose of the antigen, therefore, has been given intraperitoneally.

The route of readministration on the other hand can influence the outcome of the experiments in several ways. The speed of absorption of reinjected protein varies with the site of administration. The site of the injection determines, therefore, the time interval between the introduction of the antigen and the changes which it causes. If, furthermore, reinjection is made into sensitized shock tissue, e.g., the skin, it becomes questionable how much non-neutralized antigen reaches the rest of the animal. Accordingly, Weinberg and Seguin,<sup>17</sup> who reinjected the majority of animals intravenously and intraperitoneally, came to conclusions almost irreconcilable with those of Hajos<sup>21</sup> who administered the antigen intramuscularly or by inhalation. The intravascular reinjection affords an immediate distribution of the antigen throughout the animal and prevents its retention at the site of administration. Intracardial rather than intravenous administration was used by us because of its technical advantages, it requires, however, that a post-mortem examination be done on each animal which dies during or shortly after the injection, since the occasional perforation of the heart muscle with massive hemorrhage might simulate the asphyctic death of acute anaphylaxis.

#### *Technic of the Eosinophil Count*

The technic of preference for the study of eosinophils is the absolute eosinophil count (Zappert,<sup>20</sup> Discombe,<sup>21</sup> and Randolph<sup>22</sup>)

Unfortunately, the amount of blood which can be obtained for repeated studies from the ear veins of the guinea pig is not sufficient to make absolute blood counts as a routine procedure. This fact has disturbed various investigators (Opie<sup>15</sup>), but it has been shown that the changes in total white count during this particular type of experiment are not of sufficient magnitude to discredit the significance of findings based on changes in differential count alone. Reliability of differential counts, however, depends on uniform technical handling. The blood has to be transferred from the ear without pressure and is spread evenly into a thin film between two coverslips. Even under the most favorable circumstances, this is not always successfully accomplished. In our experiments a considerable number of preparations had to be discarded because distribution or preservation of leukocytes proved unsatisfactory. The coverslips were stained with Wright's stain and, after drying, attached to a labeled slide with Canada Balsam. Blood counts taken before reinjection of antigen and at fifteen, thirty, forty-five, and sixty minute and then at hourly intervals for twenty-four hours after reinjection of antigen demon-

strated that there is a time lag between administration of antigen and cellular response. The maximal eosinophilia was not reached before a lapse of twelve to twenty-four hours after reinjection. This observation, found in early work and re-emphasized by Hajos,<sup>21</sup> is of great theoretical interest and has not received the recognition which it deserves.

### III RESPONSE OF SENSITIZED GUINEA PIG TO REINJECTION OF SPECIFIC ANTIGEN

*Experimental problem.* It is difficult, if not impossible, to produce uniform sensitization in any given group of experimental animals. This difficulty is well known and represents one of the most serious obstacles to quantitative evaluation of results. Rich,<sup>22</sup> for instance, in his studies on the pathogenesis of rheumatic fever and periarthritis nodosa states: "This native, individual difference in reactivity, which not only determines whether a given sensitized individual will, on contact with the antigen, develop a hypersensitive reaction but also determines in what tissue the hypersensitive reaction will occur, has, in all probability, an hereditary, constitutional element." The variation in the eosinophilic response is even greater than the differences in anaphylactic reactivity; we have no indication that the mechanisms of either are related. Our experimental procedure ascertains within reasonable limits the anaphylactic antigenicity of the antigen used in each particular series of experiments. The ability of the same antigen to elicit an eosinophilic response in sensitized guinea pigs after intracardial reinjection remains to be established before an analysis of factors which influence such response can be attempted.

*Experimental procedure.* Thirty-six guinea pigs were sensitized by intraperitoneal injection of 1 cc. of horse serum without preservative. Twenty-one days later, 28 guinea pigs were reinjected with 0.75 cc. of horse serum intracardially. The 8 remaining guinea pigs were injected with 0.75 of a 1 per cent solution of crystallized ovalbumin. Differential counts were obtained of all animals prior to reinjection, of the surviving animals three hours and twenty hours after reinjection.

*Results.* The percentage of eosinophils observed twenty hours after reinjection of horse serum in a series of 12 guinea pigs is listed in column 1, table 2. None of the guinea pigs had eosinophils prior to reinjection. Subsequent to reinjection, the figures range from 0 to 26 per cent. Only one animal failed to show an eosinophilia. Only 2 out of the 8 guinea pigs injected with crystallized ovalbumin developed an eosinophilia of 2 per cent and 3 per cent respectively. Differential blood count of the remaining 6 guinea pigs showed the changes in lymphocytes which are known to follow the injection of protein, but no eosinophils. We have omitted the three hour counts from the table, since they do not add any essential information. Their possible significance will be discussed in Section V.

*Conclusions.* Preliminary experiments summarized in table 1 had shown that the nature of the antigen determines, in part, the response of the eosinophils in the sensitized and reinjected guinea pig. Response is independent of severity of anaphylactic symptoms. Regardless of its extent, however, response depends in all cases on reintroduction of the specific antigen. Injection of a nonspecific protein in sensitized guinea pigs does not result in eosinophilia. The response also varies

within a wide range in a series of animals sensitized and reinjected with the same antigen, again, the appearance of eosinophils in the peripheral circulation is a specific response whether the final level reached be low or high. Reasons for these variations are not known.

TABLE 2.—Percentage of Eosinophils Twenty Hours after Reinjection of Horse Serum in Horse Serum Sensitive Guinea Pigs

None of the animals had eosinophils prior to reinjection. Twelve guinea pigs unprotected 54 guinea pigs protected by various antihistamine drugs

Sixty four horse serum sensitive guinea pigs Thirty minutes before intracardial readministration of horse serum Injection of					
No protective drug (12 animals)	Benadryl 5 mg./Kg (12 animals)	SY 14 15 mg./kg (12 animals)	SY 18 1.5 mg./Kg (12 animals)	SY 27 15 mg./Kg (8 animals)	SY 28 15 mg./Kg (8 animals)
%	%	%	%	%	%
11	7	16	20	7	6
9	6	18	17	0	5
0	10	1	8	0	6
5	0	6	21	11	13
2	6	10	7	14	19
8	2	10	1	12	3
14	1	16	18	6	3
16	0	6	7	21	0
6	15	8	17		
1	9	1	8		
14	13	32	3		
6	12	3	9		

Averages and standard errors

8.50 ± 1.81	6.75 ± 1.50	10.6 ± 2.56	11.3 ± 1.91	8.88 ± 1.51	6.88 ± 1.40
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TABLE 2a.—Percentage of eosinophils in guinea pigs sensitized to but not reinjected with horse serum twenty hours after intraperitoneal injection of Benadryl and SY 14

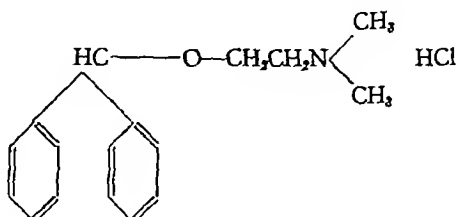
Benadryl	SY 14
1	4
3	10
0	5
0	0
1	4
0	1

#### IV RESPONSE OF THE SENSITIZED GUINEA PIG PROTECTED BY ANTIHISTAMINE DRUGS TO REINJECTION OF SPECIFIC ANTIGEN

*Experimental problem* Introduction of antihistamine drugs, the clinical significance of which is still under investigation (Feinberg<sup>14</sup>), has been instrumental in widening the scope of investigative work in allergy. It permits the study of animals of maximal sensitivity which, thus protected, survive reinjection of the specific antigen.

antigen Synthesis of compounds which combine antihistaminic and sympathetic action has made it possible to re-examine the concepts of workers who, like Hajos,<sup>21</sup> emphasize the prominence of the autonomic nervous system in eosinophilia We wish to state that this part of our study would have been incomplete, if not impossible, without the advice and the generosity of Dr E R Loew who not only supplied the necessary chemicals and the data on their comparative potency, but also consented to integrate our preliminary and final experiments on the response of the eosinophils into his own which tested the antianaphylactic action of the drugs

The first series of experiments was carried out on animals protected with  $\beta$  dimethylaminoethyl benzhydryl ether-HCl benadryl

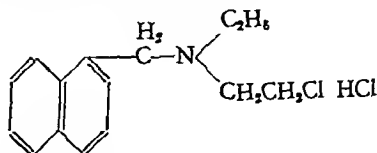


(Loew<sup>22</sup>)

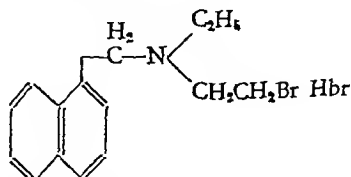
The potency of this substance is about one-twentieth of a comparable French antihistamine drug, neoantergan (N-p-methoxybenzyl-N-dimethylaminoethyl aminopyridine)

In subsequent experiments, a number of alkyl derivatives of  $\alpha$ -naphthyl-methyl  $\beta$ -chloroethylamine and of 2-bi-phenoxyethyl- $\beta$ -chloroethylamine were used \* Achenbach and Loew<sup>26</sup> have shown that the histamine antagonism produced by these compounds is of an order similar to the one afforded by benadryl and its relatives, but while the latter enhances the pressor response to epinephrine in animals, the former exert epinephrine blocking action They reverse, for instance, the pressor effect of epinephrine in dogs The following compounds were used and they are designated by their test numbers

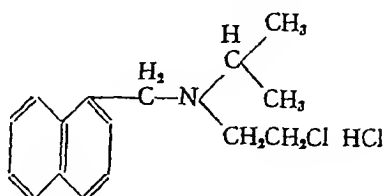
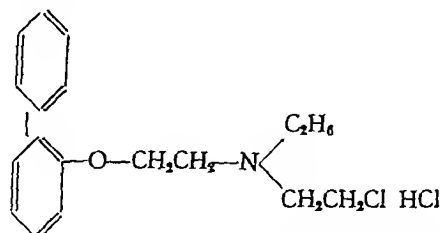
SY 14  $\alpha$ -naphthylmethylethyl- $\beta$ -chloroethylamine HCl



SY 18  $\alpha$ -naphthylmethylethyl- $\beta$ -bromoethylamine HBr



\* Those compounds were synthesized and made available to us by Drs G Rieveschl Jr R Fleming and W R Coleman of Parke, Davis and Company Detroit Michigan

SY 27  $\alpha$  naphthylmethylisopropyl- $\beta$ -chloroethylamine HClSY 18  $\beta$ -2-biphenyloxyethyl- $\beta$ -chloroethylamine HCl

Antihistamine activity of SY 14, SY 28 and SY 27 equals approximately that of neoantergan, while SY 18, like benadryl shows only one-twentieth of the protective action produced by neoantergan

*Experimental procedure* Eighty guinea pigs were sensitized with horse serum in accordance with the technic described in Section II. Twenty-one days after the sensitizing injection, they were divided into seven groups. The first group (15) was given benadryl, 5 mg /Kg in aqueous solution, thirty minutes prior to reinjection. The second group (15) was given SY 14, 15 mg /Kg in aqueous solution, thirty minutes prior to reinjection. The third group (15) was given SY 18, 15 mg /Kg in aqueous solution, thirty minutes prior to reinjection. The fourth group (10) was given SY 27, 15 mg /Kg in aqueous solution prior to reinjection. The fifth group (10) was given SY 28, 15 mg /Kg in aqueous solution thirty minutes prior to reinjection. The sixth group (10) was given benadryl, 5 mg /Kg in aqueous solution, without subsequent reinjection of horse serum, the seventh group (10) was given SY 14, 15 mg /Kg in aqueous solution without subsequent reinjection of the antigen. The antihistamine drugs were given subcutaneously or intraperitoneally. The last two groups were included in order to establish whether antihistamine drugs per se had any influence on the differential count of the guinea pig. Doses used were suggested by Dr. Loew and provided satisfactory protection against fatal anaphylactic reactions. It may be noted that the same dose was used for SY 14, SY 28 and SY 27. Quantitative studies would have to make allowance for the higher molecular weight of the bromide. Blood counts were taken three hours and twenty hours after reinjection of horse serum, or, in the last two groups, after injection of the antihistamine drug.

*Results* The percentage of eosinophils observed twenty hours after reinjection of horse serum in sensitized guinea pigs which were protected by five different antihistamine drugs against fatal anaphylactic reactions is listed in columns 3, 4, 5, and 6, table 2. The corresponding figures for the two groups of sensitized guinea

pigs which were injected with antihistamine drugs only, without subsequent re introduction of the antigen, are found in table 2a. The number of animals reported is somewhat lower than the number of those sensitized. This is due to loss of animals which for various reasons, died during or after sensitization.

The most outstanding findings in this series of experiments is the fact that the antihistamine drugs, while they block the anaphylactic reactions in the sensitized and shocked guinea pig, do not abolish the eosinophilic response. If normal distribution of the eosinophilic response could be assumed (which in view of our data is not justified) one could calculate that any difference which exists in eosinophilic response of animals protected by various antihistamine drugs is obliterated by the exceedingly high variability of such a response. This is obvious in the averages and standard errors listed in table 2a. Other types of distribution which have been tested lead to identical conclusions. Nevertheless, statistical analysis does not deny the possibility that drugs which combine histamine antagonism with sympathicolytic action, enhance the eosinophilia found in the peripheral blood twenty hours after reinjection of the antigen in sensitized guinea pigs. The administration of benadryl alone, without subsequent reinjection of the antigen, produced only minor changes in three out of six animals, the percentage of eosinophils observed after administration of SY 14 without subsequent reinjection of antigen remained below the average of reinjected guinea pigs, but showed sufficient increase to make further discussion necessary.

*Conclusions* It has been repeatedly stated that antihistamine drugs represent a tool to test, extend, or restrict the histamine theory of allergy (Mayer<sup>27</sup>), in which eosinophils have been included (Code<sup>28</sup>). Ratner<sup>29</sup> maintains that validity of the histamine concept has yet to be established. However, the fact that eosinophilic response is not abolished by antihistamine compounds lends itself to several interpretations.

If eosinophilic response is not due to release of histamine, it could not be expected to be altered by antihistamine drugs. If, on the other hand, liberation of histamine or histamine-like substances was the direct cause of appearance of eosinophils in the circulation, it must be assumed that antihistamine drugs fail to act on the underlying mechanism. This is conceivable, since various effects of histamine, e.g., its action on gastric secretion, are not uniformly influenced by antihistamine drugs. In the latter case, one could expect that eosinophilic response in protected animals would exceed the response in unprotected animals since more histamine—blocked from its pharmacologic action on smooth muscles and capillaries—might be available. It might be reasoned—paradoxical as it seems—that antihistamine drugs should exaggerate those effects of histamine against which they do not protect. As a matter of fact, the administration of antihistamine drugs alone might cause such effects as soon as the amount of histamine which they displace and divert exceeds the physiologic threshold, it is this consideration which led us to study the action of antihistamine drugs per se without subsequent re injection of antigen on the eosinophils in the guinea pig.

The mechanism of histamine activity, i.e., its rapid change from an inactive into an active form, is insufficiently understood. By the same token, the action of anti

histamine drugs has not yet been explained. We are aware that our findings that antihistamine drugs do not abolish the eosinophilia which follows an antigen antibody reaction in the guinea pig neither confirm nor disprove the possibility that histamine participates in its development.

The action of antihistamine drugs with sympathicolytic activity is of interest in view of the known fact that epinephrine decreases or abolishes an existing eosinophilia (Schwenker and Schlecht<sup>40</sup>), or reduces the eosinophilia which follows reinjection of specific antigen (Campbell<sup>24</sup>). Campbell has concluded from his experiments that epinephrine inhibits eosinophilia because it eliminates the shock syndrome without interfering with the antigen antibody reaction itself. The same statement, however, applies to antihistamine drugs which have no inhibiting effect on eosinophilic response. We feel that our concept of the importance of shock in the mechanism of the eosinophilic response requires a careful analysis and, probably, revision.

#### V. ROLE OF SHOCK IN PRODUCTION OF PERIPHERAL EOSINOPHILIA

*Experimental problem.* The importance of shock in the mechanism of the eosinophil response has been suggested by many authors, notably by Hajos<sup>21</sup> who claimed that an agitation of the autonomous nervous system, vegetative Erschütterung, is required before eosinophils appear in the peripheral circulation. E. von Neusser<sup>41</sup> had described eosinophilia following pilocarpine injection as early as 1892, Bertelli, Falta and Schweeger<sup>42</sup> demonstrated the effect of epinephrine (decrease in eosinophils) as well as that of pilocarpine and choline (increase in eosinophils), and attributed the results to action of the drugs on sympathetic and parasympathetic nerves respectively. Camp<sup>43</sup> found, however, that both parasympathetic and sympathetic stimulation raised the number of eosinophils in rabbits which makes the existence of a specific neural regulation rather unlikely.

Hajos<sup>21</sup> had concluded that the injection of foreign protein stimulates the formation of eosinophils in the bone marrow, but that an autonomic shock would force their release into the peripheral circulation. We have always felt that this concept of shock was too vague to support his theory convincingly. In order to test its validity we have grouped the experimental animals which survived the reinjection of the homologous antigen according to severity of shock symptoms. The groups were labeled as follows:

- 1 Severe shock followed by convulsions, collapse and coma ++++
- 2 Sneezing, marked dyspnea, urination and defecation ++
- 3 Chattering teeth, ruffled fur, excitement without respiratory symptoms +
- 4 and 5 Very slight, indefinite, or no symptoms  $\pm$  or 0

The results are found in table 3. In addition to our own findings, we have re-examined the figures published by Weinberg and Seguin, they list the percentage of eosinophils observed in the blood of guinea pigs sensitized with horse serum twelve to twenty-four hours after subcutaneous or intraperitoneal reinjection. Table 3a lists those guinea pigs which had no eosinophils prior to reinjection and are, therefore, comparable to our own, the symptoms were "marked" but no description in detail was given, and we have assumed that they correspond roughly



to our second, ++, group Table 3b lists those of Weinberg and Seguin's<sup>17</sup> animals which had eosinophils ranging from 4 to 17.6 per cent in the peripheral blood

TABLE 3—*Severity of Shock Symptoms and Eosinophilia (35 guinea pigs)*

++++	Severe shock followed by convulsions collapse coma.
++	Sneezing marked dyspnea, urination defecation.
+	Chattering teeth, ruffled fur excitement without respiratory symptoms
± 0	Very slight, indefinite or no symptoms

++++	++	+	±	0
6	5	6	21	3
11	9	6	11	7
10	0	17	14	12
2	2	7	3	1
0	7	6	12	21
9	20	8	6	0
7	8	0	6	6

Percentage of eosinophils (twenty hours after reinjection) of guinea pigs sensitized and re-injected with horse serum. None had eosinophils prior to reinjection.

TABLE 3a—(Compiled from Weinberg and Seguin<sup>17</sup>)

Shock symptoms and eosinophils

No symptoms	++
2.6	0
4.3	0.6
3	1.6
3.3	2
2.6	2.3
17.3	8.3
0	1
9.3	6
12.3	18
2	5.3
5.6	

Percentage of eosinophils (twelve to twenty four hours after subcutaneous or intraperitoneal reinjection) in guinea pigs sensitized and re-injected with horse serum. Percentage prior to reinjection less than one per cent.

TABLE 3b—(Compiled from Weinberg and Seguin<sup>17</sup>)

Shock symptoms and eosinophils

Before reinjection	After reinjection	Route	Symptoms
4	8.3	s.c.	none
5.3	18	s.c.	none
8	27.6	s.c.	none
9	19.3	s.c.	none
5.3	17.6	i.p.	++
4	16	i.p.	none
15.6	14.3	i.p.	++
17.6	29	i.p.	none
14.6	22.6	i.p.	none
6	12.6	i.p.	++
9.6	16.3	i.p.	++
14	14	i.p.	++

Percentage of eosinophils (twelve to twenty four hours after reinjection) in guinea pigs sensitized and re-injected with horse serum.

prior to reinjection of horse serum. We have eliminated from our experiments animals which showed an eosinophilia prior to the "shocking" injection because the introduction of another variable factor would further complicate the already

difficult interpretation. In this instance, however, table 3b illustrates well the point in question of the 12 animals listed, 5 had marked symptoms of shock, 7 had none, the fact that only the intraperitoneal, not the subcutaneous, reinjection produced symptoms is of course a phenomenon with which investigators have long since become familiar.

*Results and conclusions.* The tables are self-explanatory and the conclusions are evident. We have been unable to demonstrate any correlation between intensity of shock symptoms and appearance of eosinophils in the peripheral circulation of guinea pigs sensitized and reinjected with horse serum. Our findings compare well with those established by previous authors in experiments which were conducted for different reasons, but employed a technic comparable to ours. They are also in accord with the findings summarized in table 1, where reinjection of hapamine produced a more pronounced eosinophilia in sensitized guinea pigs than ovalbumin, although the anaphylactic symptoms caused by reintroduction of hapamine were much less severe than those which followed the reinjection of ovalbumin.

#### VI. RESPONSE OF NONSENSITIZED AND SENSITIZED GUINEA PIGS TO INJECTION OF SUBSTANCES LIBERATED DURING ANTIGEN-ANTIBODY REACTION HISTAMINE PHOSPHATE, HEPARIN, ADENOSINE

*Experimental problem.* It had been recognized early that while homologous antigen, protein, is instrumental in the course of events which result in peripheral eosinophilia, it is not its direct cause. Schlecht<sup>26</sup> had examined the ability to produce eosinophilia of a considerable number of substances including leucine, alanine, phenylalanine, glycocoll, asparagine, and also sugar, starch and olive oil. All of these failed to produce an eosinophilic response. In view of the complexity of the literature on the subject, it seems necessary to point out that the chemicals which have been tested fall into two categories: those which act, and those which fail to act on vasomotor regulatory mechanisms. It is important to distinguish clearly between the two groups, a considerable portion of described changes might be attributed to a shift in distribution of the corpuscular elements of the blood. Dobreff, Doitschneff and Marinoff<sup>27</sup> have coined the term "Verteilungsleukocytose" for this phenomenon, and—to name a practical application—we suspect that Vaughan's<sup>28</sup> so-called leukopenic index might be explained on a similar basis. Drugs which stimulate or inhibit autonomic nerves, belong to the first group, e.g., epinephrine, atropine, physostigmine, acetylcholine, histamine, substances like sugar or cysteine belong to the other.

The distinction which we have just outlined is important for analysis of factors which participate in development of peripheral eosinophilia. It is possible that the eosinophilic response is controlled by vascular mechanisms, e.g., the afferent and efferent circulation of the bone marrow. Part of the experimental evidence points in this direction. Yet, there are other possibilities to be considered. It is conceivable that the eosinophilic response is due to chemical action on a specific enzyme system, vascular factors might, secondarily, control its intensity. The anaphylactic reaction is complex and involves a variety of biologic changes, such as the contraction of smooth muscles in shock tissues, the decreased coagulability of the

blood, the eosinophilic response Dragstedt<sup>16</sup> has concluded that separate substances are responsible for at least two of these manifestations. The evidence must be considered conclusive that a tissue liberation of histamine, of heparin, and possibly of choline occurs during the anaphylactic reaction in various animals. In the dog the liberation of heparin can completely account for the incoagulability of the blood and there is no reason to doubt that it may be found in other animals. Accordingly, the eosinophilic response again might be caused by a different compound which is not yet defined. It is possible that the distinction which we have suggested will facilitate its eventual identification.

The compounds mentioned by Dragstedt appear subsequently to the antigen antibody reaction. Since most of the substances occur under physiologic conditions in the experimental animal, pathologic changes will not result unless the amount injected exceeds the threshold of physiologic balance. We are unable to predict this threshold with regard to the eosinophilic response, accordingly, conclusions must be restricted to the route and the amount used in each experiment.

As far as we have been able to ascertain, no previous studies have been made of the effect on eosinophilia of adenosine and heparin. There is evidence that either might be released during anaphylactic reactions (Rocha e Silver<sup>17</sup>). Adenosine has a rather weak dilating effect on peripheral arterioles, but administered subcutaneously, [it] causes a migration of leukocytes to the site of injection, an effect which is not produced by histamine (Best and Taylor<sup>18</sup>).

Experimental studies by Campbell<sup>24</sup> demonstrated that guinea pigs which were given 0.5 mg. of histamine, three times a day for three consecutive days, showed no increase in the percentage of eosinophils during five days following the first injection. He found, however, that guinea pigs sensitized and reinjected with ascari keratin responded with a more marked increase in eosinophils when the reinjection of the antigen was followed by a series of histamine injections as previously described. The same effect could be obtained, however, if acetylcholine and cysteine, instead of histamine, were used. It is difficult, therefore, to interpret Campbell's findings. He used substances of different biologic activity and we cannot think of any common denominator. The use of antihistamine drugs in our experiments made it possible to inject an amount of histamine which was comparable to the level of histamine or histamine-like substances liberated during anaphylactic reactions, an amount which would be fatal without this protection, Campbell, in his experiments administered histamine intraperitoneally and did not describe any symptoms of shock such as would have to be expected if similar doses were given intracardially.

If nonantigenic compounds fail to call forth an eosinophilic response, the negative results might be due to the ineffectiveness of the given chemical agent, or to the fact that the injected animal was one of those incapable of producing eosinophilia no matter what the stimulus. We will have to rule out the latter possibility by reinjection of the specific antigen subsequent to the injection of the compound under investigation. Exceptions to this rule would be permissible only if it were possible to obtain a strain of guinea pigs which would afford, under standard experimental conditions, a uniform eosinophilic response. We have

discussed the problem with Dr D H Campbell, whose work seemed to indicate that his experimental animals responded more homogeneously than ours to sensitization and reinjection with a variety of antigens Dr Campbell was kind enough to supply us, for breeding purposes, with several animals from his strain which has been inbred for more than five years We intend to study their response as soon as a sufficient number of animals are available

*Experimental procedure* Guinea pigs (32) were sensitized with horse serum in accordance with the technic outlined in Section II Twenty-one days after the

TABLE 4.—Percentage of eosinophils in the peripheral blood of horse serum sensitive guinea pigs before, three hours and twenty hours after intracardial injection of adenosine, heparin and histamine phosphate respectively Percentage of eosinophils in the peripheral blood of the same animals before three hours and twenty hours after intracardial reinjection of horse serum seventy-two hours later

Guinea Pig No	Intracardial inj of adenosine, % of eosinophils			Intracardial inj of heparin % of eosinophils			Intracardial inj of histamine % of eosinophils			Intracardial inj of horse serum % of eosinophils		
	bef inj	3 hrs after inj	20 hrs after inj	bef inj	3 hrs after inj	20 hrs after inj	bef inj	3 hrs after inj	20 hrs after inj	bef inj	3 hrs after inj	20 hrs after inj
108	1	1	0							0	3	6
111	0	0	0							0	2	11
114	0	0	1							0	3	9
132	0	1	0							0	4	5
145	0	1	0							1	3	14
147	0	0	0							0	7	26
363				0	1	2				0	2	8
376				0	1	0				0	4	5
378				0	0	0				1	3	3
383				0	0	3				0	4	12
384				0	1	4				1	2	4
385				0	2	5				0	5	5
359							0	3	17	0	11	13
361							7	0	1	6	3	14
362							0	3	5	4	3	14
417							0	2	0	0	0	1
449							0	4	8	3	2	10
453							0	2	9	4	9	15
456							0	3	6	—	6	9
457							0	2	5	2	5	13

sensitizing injection, they were divided into three groups The first group (8) was injected with adenosine, 1 mg in 0.5 cc of distilled water, intracardially The injection of adenosine was followed seventy-two hours later by the intracardial reinjection of 0.75 cc of horse serum The second group (8) was injected with heparin, 1 mg in 0.5 cc of distilled water intracardially, the injection of heparin was followed seventy-two hours later by the intracardial injection of horse serum as described before The third group (16) was injected with histamine phosphate 0.5 mg/Kg in 0.5 cc of distilled water intracardially, 1 mg of this solution represents 0.36 mg histamine base The injection of histamine phosphate was followed

seventy-two hours later by the reinjection of 0.75 cc of horse serum intracardially as described before. At the same time, a group of nonsensitized guinea pigs (12) were given histamine phosphate, 0.5 mg/Kg in distilled water, intracardially.

Differential counts were taken before and three hours and twenty hours after each intracardial injection. The animals were given benadryl, 5 mg/Kg in aqueous solution, intraperitoneally, thirty minutes prior to the injection of histamine phosphate and horse serum, adenosine and heparin were injected without protection. Benadryl was selected as the antihistamine drug for this series of experiments, because we had previously shown that, *per se*, it failed to increase the percentage of eosinophils in the sensitized guinea pig.

**Results.** The results of the injections of adenosine, heparin and histamine followed by the reinjection of horse serum are listed in table 4. Adenosine, in the amount injected did not produce an increase in eosinophils, the increase observed after the injection of heparin is not significant enough to warrant conclusions, it might be caused, for instance, by a minute amount of impurities which adhere even to highly purified preparations.

A considerable number of animals injected with histamine died immediately after or during the first twelve hours following the injection. There was, however, no apparent difference in tolerance between the non-sensitized and sensitized group. The non-sensitized animals had a differential count of 0, 0, 1, 2, 3 and 6 per cent eosinophils, respectively, twenty hours after the intracardial injection of histamine phosphate. The percentage of eosinophils in the sensitized group range from 0 to 17 per cent, 6 of the 8 animals, however, which are included in this group, had an eosinophil count of 5 per cent or more.

The reinjection of horse serum makes it evident that animals No. 417 and No. 449 must be eliminated from the series for both failed to respond to the subsequent reinjection of the specific antigen. Animal No. 361 has been included in order to emphasize the fact that the response of the guinea pig to any given substance is obscured if an eosinophilia is present prior to the experiment, this is true even if the response to subsequent reinjection of the specific antigen is satisfactory.

Table 4 also lists the differential eosinophil count obtained three hours after intracardial injection. We are unable as yet to explain the variation in speed with which the maximum percentage of eosinophils is reached. Animals No. 359 and No. 362, for instance, had 3 per cent eosinophils after three hours, but the former showed 17 per cent, the latter only 5 per cent after twenty hours. Similarly, the return to the pre-experimental level varies considerably. The behavior of the eosinophils in animals which showed an eosinophilia prior to the reinjection of the specific antigen, presents another interesting aspect of the same question. We have listed, in table 5, two groups of guinea pigs sensitized and reinjected with horse serum. The first group had not more than 1 per cent, the second group from 2 to 7 per cent eosinophils before the shocking injection of horse serum was given. A comparison of the percentage of eosinophils observed in each group three hours and twenty hours after reinjection, makes us suspect that a balance develops between an initial disappearance and a secondary reappearance of eosinophils in the

blood stream Differential counts at short intervals might provide a definite answer, they would extend, however, our study beyond its present scope

*Conclusions* The observation that histamine per se is able to produce an increase in eosinophils in the blood of sensitized guinea pigs, might explain Campbell's findings that it enhances the eosinophilic response which follows the reinjection of homologous antigen We hesitate on the other hand to draw any more far-reaching conclusions as to the underlying mechanism, since Campbell reports even a larger increase by the use, in a corresponding technic, of acetylcholine and cysteine

## VII CORRELATION OF EOSINOPHILS IN BONE MARROW, PERIPHERAL CIRCULATION AND SHOCK TISSUE

*Experimental problem* It had been recognized and stated by early investigators that any attempt to understand the mechanism of peripheral eosinophilia requires the simultaneous study of bone marrow, peripheral circulation and shock tissue Curiously enough, no accord has been reached about the interpretation of findings Weinberg and Seguin,<sup>17</sup> for instance, found in a study of eosinophils in the blood and lungs of guinea pigs that 4 out of 10 animals sensitized but not reinjected with horse serum as well as 9 out of 22 animals sensitized and reinjected with horse serum had eosinophils in their lungs provided they had a high eosinophil count in their blood They concluded that the presence of eosinophils in the lungs had no relation to anaphylaxis and termed the phenomenon chronic spontaneous eosinophilia

Hajos,<sup>21</sup> on the other hand, using the same species and the same antigen, based his conclusions on the examination of bone marrow, peripheral blood count and lungs of guinea pigs sensitive to horse serum prior to reinjection and from eight to forty-eight hours after reintroduction of the specific antigen by intramuscular injection or by inhalation He found that of the three groups examined only those which were re-exposed to the antigen by inhalation showed a pulmonary eosinophilia Homma,<sup>18</sup> who used white rats injected with parasites and parasitic material, felt confident that he had established a direct relationship between eosinophils in bone marrow, blood and shock tissue He maintained that eosinophils increase in the bone marrow during sensitization and, furthermore, that he had been able to correlate their subsequent decrease in the bone marrow with their increase in the peripheral circulation and their final decrease in the peripheral circulation with their increase in the shock tissue The cycle thus established would confirm Ehrlich's concept of the origin and distribution of eosinophils Unfortunately, his paper contains neither figures nor a description of his methods, and the Japanese journal to which he refers for the details of his experimental procedure is not available to us It is therefore impossible to reproduce his experiments For this and other reasons mentioned earlier, we decided to study bone marrow and shock tissues of a series of guinea pigs which were sensitized and reinjected with horse serum by the standard procedure employed throughout our investigation

The study of bone marrow presented us with a number of technical problems. Comparative studies convinced us that of the three available methods, marrow puncture, touch preparation of marrow, and marrow section, the last gave the most consistent results. The number of eosinophils within the area of a grid was the technic adopted \*

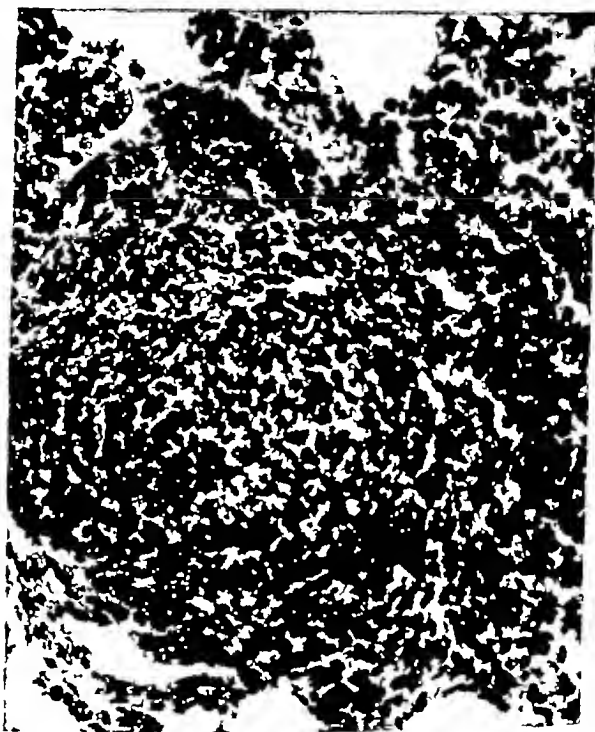


FIG. 1 Eosinophils accumulated in the periphery of an intrapulmonary lymph node of a guinea pig sensitized and reinjected with horse serum twenty hours after reinjection. Hematoxylin azure-eosin. Magnification  $1 \times 400$

The examination of shock tissues does not offer any particular difficulties. The lungs which represent the essential shock tissue in the guinea pig were classified into six groups according to the number of eosinophils observed

- no eosinophils o
- few eosinophils (one or two per h p f) +
- moderate eosinophilia ++
- marked eosinophilia +++
- massive eosinophilia ++++

This corresponds roughly to the classification used by Weinberg and Seguin<sup>17</sup>

\* We are indebted to Dr. L. R. Lamarca for his suggestions and his help in identifying the preparations.

The eosinophils are seen to accumulate in the perivascular and peribronchial connective tissue. They are prevalent in septal tissue and bronchial walls and are imbedded in mucus in the lumen of the bronchi. As a rule, a large number surround the intrapulmonary lymphatic tissue which occurs normally in guinea pigs, and while a few are observed in lymphatic channels, the major portion is found in the periphery of the lymphnodes (fig 1), a relationship which had been described by Opie<sup>15</sup> more than forty years ago.

*Experimental procedure* Guinea pigs (18) were sensitized to horse serum by the method previously described. Twenty-one days later, they were divided into three groups of 6 animals each. One group was given 0.75 horse serum intracardially without protection, the second group received benadryl, 5 mg/Kg in aqueous solution, the third group SY 18, 1.5 mg/Kg in aqueous solution intraperitoneally, thirty minutes prior to the intracardial reinjection of horse serum. Three of the unprotected animals and two of the animals protected with SY 18 died from the result of immediate or delayed anaphylactic reactions.

Another series of guinea pigs (6) was sensitized by intraperitoneal injection of 0.75 cc of 1 per cent ovomucin in aqueous solution. Twenty-one days later, the guinea pigs were divided into three groups of two animals each. One group was given 0.75 cc of a 1 per cent aqueous solution of ovomucin intracardially without protection, the second group received benadryl, 5 mg/Kg in aqueous solution, the third group SY 18, 1.5 mg/Kg in aqueous solution, thirty minutes prior to the reinjection of ovomucin. One of the unprotected animals died from the results of immediate anaphylactic shock.

None of the animals had more than 1 per cent eosinophils prior to reinjection. Twenty hours after the intracardial reinjection, differential counts were obtained in all the animals, and in four nonsensitized guinea pigs. After the counts had been taken, the animals were sacrificed. The first animals were sacrificed by fracturing their necks. In view of the marked extravasation of blood which results from violent death, this procedure was abandoned and the majority of the animals were sacrificed by intracardial injection of nembutal in aqueous solution. Immediately after death, postmortem examinations were performed, specimens were removed of lungs and of any organ which showed gross pathologic changes. One femur was carefully opened, the bone marrow penciled out in toto. The specimens were fixed in Zenker-Formol, freshly prepared. They were imbedded in paraffin and stained with hematoxylin-azure-eosin. This stain permits a satisfactory identification of eosinophils in all preparations. A grid inserted into the ocular of the microscope was used for the counting of eosinophils in the bone marrow. The figures thus obtained are relative figures and not comparable with those quoted by other authors.

*Results* The correlation of eosinophils in bone marrow, peripheral circulation and lungs is summarized in table 6.

1. Bone marrow. Eosinophil counts in the bone marrow of non-sensitized guinea pigs varied from 8 to 52 in the area of the grid used during this study, in the bone marrow of guinea pigs sensitized and reinjected with horse serum, from 7 to 62 in the bone marrow of guinea pigs sensitized and reinjected with ovomucin, from



TABLE 5—Percentage of eosinophils (before, three hours and twenty hours after reinjection) in guinea pigs sensitized and re injected with horse serum

The first group had 1% or less, the second group from 2% to 7% eosinophils prior to reinjection  
 1st group twelve guinea pigs without eosinophils prior to reinjection      2nd group twelve guinea pigs with eosinophils present prior to reinjection.

% of eosinophils			% of eosinophils		
before reinj	3 hrs after reinjection	24 hours after reinjection	before reinj	3 hrs after reinjection	20 hours after reinjection
0	2	5	6	2	14
0	3	9	3	6	9
0	0	6	3	3	13
0	9	17	2	9	2
0	3	7	2	3	5
0	3	6	7	4	2
0	2	10	4	5	3
0	8	21	3	1	3
0	4	14	5	4	8
1	8	11	3	10	2
1	2	3	3	0	1
1	1	1	6	7	14

TABLE 6—Correlation between eosinophils in bone marrow peripheral blood and shock tissue in normal controls and guinea pigs sensitized and re injected with horse serum and ovomucin twenty hours after reinjection

No	Antigen	Symptoms	Eosinophils			Anti histamine drug used
			Bone marrow	Periph count	Lungs	
317	Horse serum	0	19	2	0	SY 18
318	Horse serum	0	52	0	+	SY 18
321	Horse serum	+	62	17	++++	SY 18
323	Horse serum	0	24	7	+	none
324	Horse serum	+	29	1	0	none
325	Horse serum	0	7	0	0	none
328	Horse serum	0	28	6	+	Benadryl
329	Horse serum	±	22	12	+++	SY 18
330	Horse serum	0	25	1	++	Benadryl
331	Horse serum	+	15	19	++	Benadryl
332	Horse serum	0	12	5	++	Benadryl
333	Horse serum	0	17	10	++	Benadryl
335	Horse serum	0	?	2	+	Benadryl
316	Ovomucin	0	83	3	+	Benadryl
336	Ovomucin	0	18	1	0	Benadryl
347	Ovomucin	0	29	16	+++	SY 18
344	Ovomucin	0	55	2	0	non
358	Ovomucin	0	68	1	+	SY 18
411	—	—	52	0	+	Control
416	—	—	15	0	++	Control
441	—	—	8	0	0	Control
442	—	—	10	0	0	Control

18 to 83. The average of the animals reinjected with horse serum is slightly higher, and of those reinjected with ovomucin considerably higher, than the average of nonsensitized controls. We believe that the difference between nonsensitized and sensitized animals is likely to be more pronounced before rather than after the reinjection which causes a redistribution of eosinophils. Two animals must be classified as experimental failures: guinea pig No. 325 failed to respond to sensitization and reinjection with horse serum, it showed for unexplained reasons



FIG. 2. Eosinophilia in the lung of a guinea pig sensitized and reinjected with horse serum, twenty hours after reinjection. Benadryl, 5 mg/Kg intraperitoneally thirty minutes prior to reinjection. Hematoxylin azure-eosin.

neither anaphylactic symptoms nor tissue reactions. Guinea pig No. 335 is listed without its bone marrow count since a technical mishap made the specimen unfit for interpretation.

The variation between the eosinophil counts in the bone marrow of individual guinea pigs is so marked that the examination of the bone marrow does not permit any conclusion as to whether it has been obtained from a non-sensitized animal or from an animal which has been sensitized and reinjected with the specific antigen. We have not been able to establish any correlation between the number of eosino-

phils in bone marrow and peripheral blood. Five guinea pigs which had peripheral counts of 10, 12, 16, 17 and 19 per cent respectively, had, in the corresponding order, bone marrow counts of 17, 22, 29, 15 and 62 eosinophils in the grid area.

2. **Lungs** Of the nonsensitized controls, eosinophils were absent in 2 specimens, one showed a few eosinophils, one a moderate number and none of the controls had eosinophils in their peripheral blood at the time of death. Of the guinea pigs sensitized and reinjected with horse serum, 3 failed to show eosinophils in the lungs, 4 showed a few eosinophils, 4 had a moderate, 1 a marked and 1 a massive eosinophilia. Of the guinea pigs sensitized and reinjected with ovomucin, 2 failed to show eosinophils, 2 showed a few eosinophils, 1 had a marked eosinophilia. Of the 5 animals which showed a moderate eosinophilia in their lungs, 3 had a peripheral eosinophil count of 5 per cent or more, the animals (3) which had a marked or massive pulmonary eosinophilia, had peripheral eosinophil counts of 12, 16 and 17 per cent. With the exception of the control (guinea pig No. 416), the animals which displayed a moderate pulmonary eosinophilia, had been protected with benadryl, those which showed a marked or massive eosinophilia, with SY 18. On the other hand, eosinophils were absent in one of the animals treated with benadryl, one of the animals treated with SY 18, a few eosinophils were seen in the remaining animals of either group. Anti-histamine drugs did not abolish the eosinophilic response in the lungs of guinea pigs sensitized and reinjected with antigen. Figure 2 shows the histologic section of the lung of guinea pig No. 332 protected by benadryl.

A definite correlation appears to exist between peripheral eosinophilia and shock tissue. All of the animals which had more than 10 per cent eosinophils in the peripheral blood, showed a moderate, marked, or massive number of eosinophils in their lungs.

**Discussion** 1. Bone marrow Hajos<sup>21</sup> who tried to correlate eosinophilia in bone marrow, blood and lungs, found percentages of 1.3 per cent and 1.5 per cent of cells with eosinophilic granules—myelocytes, myeloblasts and mature cells—in the bone marrow of normal guinea pigs. He counted a total of 1000 bone marrow cells—a technic comparable to our own. Accepting these figures as a base line, he proceeded to examine the bone marrow of guinea pigs sensitized to horse serum. The percentage of eosinophils increased to a maximum of 9 per cent, while the peripheral eosinophil count failed to rise. Intramuscular reinjection of the specific antigen decreased the percentage of eosinophils slightly, inhalation of the specific antigen considerably. The differential count of the bone marrow, eight to twenty-four hours after exposure of the sensitized guinea pig to nebulized horse serum, ranged from 1 per cent to 3 per cent. At the time of the decrease in eosinophils of the bone marrow, a slight increase was noted in the peripheral blood, it did not exceed, however, 4 per cent in the latter group. We have been unable to confirm the existence of a normal eosinophil count in the bone marrow of guinea pigs. The pronounced variation, in our opinion, is explained by the actual difference in the number of eosinophils present in the bone marrow of each individual animal, but is enhanced by the characteristics of distribution of eosinophilic cells within the same bone marrow. Figure 3, a bone marrow section of guinea pig No. 411,

illustrates the irregularity of the pattern which becomes even more significant when the percentage of eosinophils is low. The latter consideration is of minor importance. Examination of several sections and increase of the total number of cells counted might establish a valid average. While it might thus be possible to observe increase or decrease of cells with eosinophilic granulation in the same animal, we do not believe that changes in the percentage of eosinophils in the bone marrow of different guinea pigs are comparable.



FIG. 3 Eosinophils in the bone marrow of a non-sensitized guinea pig. Hematoxylin azure-eosin. Magnification  $1 \times 400$ .

2. Lungs. The study of eosinophils in shock tissues has assumed renewed importance, since Halpern<sup>49</sup> reported that the injection of antihistamine drugs derived from thiodiphenylamine prevented the eosinophilia usually found in the lungs of guinea pigs twenty-four hours after the anaphylactic reaction. Halpern, in his experiments, used sheep serum as antigen, the sensitizing dose was given intraperitoneally in two portions, three days apart, the shocking dose, fifteen to twenty-one days later into the jugular vein. He states that this technic causes fatal shock in 100 per cent of unprotected animals. Its anaphylactic antigenicity appears to be superior to the horse serum used in our experiments, we have no information about the peripheral eosinophilic response in the animals thus sensitized. If the series

of antihistamine drugs studied by Halpern prevents the development of an eosinophilia in shock tissues, while the type of preparations which we have used either fails to influence or even increases the number of eosinophils present, the difference thus established should provide a clue of fundamental significance

3 Correlations The concept of chronic spontaneous eosinophilia which we have mentioned in the beginning of this section has been developed by Weinberg and Seguin<sup>17</sup> as the result of comparative studies of blood and lungs in sensitized guinea pigs before and after reinjection of the specific antigen. We have no cause to doubt the validity of their findings, but we are certain that the observations upon which the concept is based require re-evaluation, because a large number of the experimental animals had a high peripheral eosinophil count prior to reinjection. The importance of this distinction becomes evident, if we analyse the factors which might influence the number of eosinophils in blood and shock tissue. We have reason to assume that a specific stimulus such as originates during the antigen antibody reaction is necessary to produce a peripheral eosinophilia. Opie,<sup>18</sup> Weinberg and Seguin,<sup>17</sup> and recently Ingraham and Wortman<sup>19</sup> have demonstrated beyond doubt, however, that the chemotactic behavior of eosinophils equals that of neutrophils, that they are, moreover, phagocytic in vitro and in vivo. Accordingly, if they are once present in the blood stream, a variety of pathological changes might account for their presence in various tissues, this is one of the considerations which has caused us to restrict our studies to guinea pigs which had no peripheral eosinophilia prior to reinjection of the specific antigen.

The second factor which might influence the distribution of eosinophils in blood and shock tissue is even more pertinent because it applies to our own experiments in which the antigen antibody reaction was the immediate and undisputable cause for appearance of eosinophils in the circulation. Our studies seem to confirm Weinberg and Seguin's observations which correlate peripheral and pulmonary eosinophilia. Yet it has to be pointed out that this apparent correlation might be seriously distorted. Gerlach<sup>20</sup> in his classic experiments had emphasized the importance of factors which determine where the antigen antibody reaction is localized. The lungs represent only one of the shock tissues of the guinea pig; the entire gastrointestinal tract, bladder, uterus, and skin are bound to participate in antigen antibody reactions and to influence the distribution of eosinophils. It is quite likely, as a matter of fact, that the total number of eosinophils present in shock tissues, other than lungs, might be considerably larger than the pulmonary fraction. Figure 4 represents a typical example: a section of the stomach of guinea pig No. 323 which was sensitized and reinjected with horse serum. The guinea pig, although not protected by antihistamine drugs, did not exhibit anaphylactic symptoms, it was sacrificed twenty hours after reinjection. It had a peripheral eosinophil count of 7 per cent at the time of its death. Only few eosinophils are seen in its lungs, the stomach, on the other hand, shows marked edema and a massive eosinophilia. It is obvious that in the representative case of the animal which we have just described, a comparative study which disregards the gastric reaction would be of doubtful value. We were impressed by the apparent affinity of eosinophils to the connective tissue of the specimens which we have examined.

It is possible that the eosinophilic response might require the interaction of the antigen with the tissue during the antigen-antibody reaction with a connective tissue component in the organs. We have no concept of the possible manner in which the connective tissue might participate in the



FIG. 4. Eosinophils in the lamina propria of the stomach of a guinea pig, sensitized and re injected with horse serum twenty hours after reinjection. Hematoxylin azure-eosin. Magnification  $1 \times 100$ .

process of sensitization, but we suspect that further studies of the function of eosinophils will also uncover a specific function of the connective tissue which has, so far, escaped our attention.

While we feel that the results of our studies on correlation are of interest with regard to the action of antihistamine drugs, we are also aware of the fact that the actual problem of the mechanism which correlates the presence of eosinophils in blood and lungs has not been solved. It is not possible to decide by static in-

vestigations such as ours and those of previous workers whether the increase of eosinophils in the blood is the cause or the result of the eosinophilia observed in shock tissue. It might be necessary to study the eosinophilic response in shock organs which are isolated *in vivo* and permit the continuous determination of the percentage of eosinophils in the arterial and venous circulation with which they are supplied. In humans, the investigation of the eosinophilic response in shock tissues has been confined to the skin.

Kline, Cohen and Rudolph<sup>52</sup> found a marked eosinophilic infiltration in the skin of allergic individuals, twenty to twenty five minutes after injection of either histamine or specific antigen. The initial count of 50 per cent decreased to about 10 per cent after three hours. Nonallergic persons failed to exhibit this transitory local eosinophilia, the injection of histamine caused only a slight inflammatory reaction.

Jadassohn<sup>53</sup> on the other hand described local eosinophilia in the human skin after mechanical irritation injection of morphine, atropine or pilocarpine even in the absence of blood eosinophilia.

Knott and Pearson<sup>54</sup> demonstrated in similar studies that the site of a positive skin test in allergic individuals contains twenty minutes after injection of the antigen approximately twice as many eosinophils as the peripheral blood, the site of a negative skin test in allergic individuals as well as in normal controls, a number which equals the peripheral count. The injection of histamine causes a local eosinophilia in the wheals formed in either nonallergic or allergic individuals. It is twice as high as the peripheral count in the former two and one-half times as high in the latter.

It must be assumed that the discrepancies in the findings of the three authors are due to a lack of uniformity in those factors which determine genesis and extent of local eosinophilia, e.g., in type and degree of individual sensitivity. We hope that the controlled conditions of the animal experiment will permit us to arrive at conclusions which clarify the open question about the agents responsible for the presence of eosinophils in shock tissues.

### VIII. CONCLUSIONS

Our results appear to have clarified a number of questions which have obscured the investigation of the eosinophilic response. It has been shown that discrepancies found in the literature are largely due to variations inherent in the nature of the antigen, its route of administration and variations in the responsiveness of the experimental animal. We have standardized our experimental procedure to eliminate as many variables as possible. Shock *per se* does not seem to account for the eosinophilia which develops subsequent to the reinjection of the specific antigen in sensitized guinea pigs. The eosinophilic response, unlike the anaphylactic reaction, is not abolished by the antihistamine drugs which we have used. Observation of eosinophils in the blood and tissue of animals thus protected suggest that there might be important differences between the various types of antihistamine drugs which are now available. We have, finally, analyzed the possible correlation between eosinophilia in bone marrow, peripheral circulation and shock tissue. Although such correlation has been found to exist in several instances, we have also come to realize the limitations which prohibit far reaching conclusions.

It might be permissible to discuss briefly possible avenues of future approach. In view of the fact that eosinophils appear subsequent to sensitization and reinjection of antigen, several investigators have attempted to relate the function of the

eosinophil to the antigen antibody reaction and to reproduce its assumed action *in vitro*. All these experiments have remained inconclusive.

Weinberg and Seguin<sup>17</sup> for instance sensitized guinea pigs with repeated intraperitoneal injections of hydatid fluid and obtained a peritoneal exudate rich in eosinophils. The cells were washed, counted, resuspended in a measured amount of hydatid fluid and incubated. Complement fixation tests on the fluid before and after incubation revealed a loss of antigen proportional to the number of eosinophils in the exudate. The experimental technique used by these authors, however, permits interpretations other than those proffered; we hesitate to accept their conclusion that the antigen has been absorbed by the eosinophils which they suggest might produce specific antibodies after absorption.

Three recent publications refer to *in vitro* experiments on the function of eosinophils in the mechanism of antigen antibody reactions. Ringo<sup>20</sup> writes: "Olson's recent studies of the eosinophils in immune reactions indicate that a specific sensitizing product is formed between eosinophilic leukocyte granules and complex proteins." Osgood<sup>21</sup> with Perlman studied the development of eosinophils in bone marrow cultures. Eosinophils formed when the specific allergen was added to cultures of the marrow of allergic patients. They did not develop in cultures of bone marrow of nonallergic individuals which had been sensitized by addition of a small amount of allergic serum. Histamine did not produce eosinophilia in either allergic or nonallergic cultures. Kirk<sup>22</sup> finally reports: "Dr. Houghton in tissue cultures of cells from normal adults plus the serum of sensitive individuals produced an increasing number of developing eosinophils; likewise the juvenile and adult eosinophilic cells lived longer." In an attempt to secure additional details we have communicated with each of the authors, unfortunately—in part due to circumstances beyond control—none has completed the work beyond this suggestive stage.

While it is conceivable that *in vitro* studies might result in the sudden discovery of the function of the eosinophil, *in vivo* experiments will accomplish the same objective by a steady process of elimination and change. We believe as Campbell<sup>24</sup> does that whatever the function of the eosinophil may be it is the same under all conditions. Of several theories, however, which have been advanced to explain the presence of eosinophils in blood and shock tissues after antigen antibody reactions, none has been confirmed.

## IX. SUMMARY

1. The eosinophilic response of the guinea pig sensitized and reinjected with the specific antigen varies with the nature of the antigen used, but also with the individual guinea pig in any group sensitized and reinjected with the same antigen.
2. Certain antihistamine drugs which abolish anaphylactic symptoms, do not abolish the eosinophilic response.
3. The severity of anaphylactic shock symptoms has no influence on the eosinophilic response.
4. Histamine phosphate has no effect on the eosinophil count of nonsensitized guinea pigs; protected by benadryl, it causes a distinct eosinophilic response in sensitized animals.
5. Heparin—in the dose injected—produced only an insignificant rise in the peripheral eosinophil count of sensitized guinea pigs; adenosine had no effect.
6. Attempts were made to correlate the eosinophilic response in bone marrow, blood and shock tissue of guinea pigs sensitized and reinjected with a specific antigen. The variation within a wide range of the number of eosinophils in the bone marrow of nonsensitized and of sensitized, reinjected guinea pigs is emphasized. A definite correlation seems to exist between the presence of a large number of



eosinophils in blood and lungs, it is shown, however, that this observation permits only limited conclusions

7 The factors which account for discrepancies in the interpretation of the eosinophilic response, e.g., nature of antigen, route of administration and characteristics of species, are analyzed

8 The significance of the findings is reviewed in the light of previous work

#### ACKNOWLEDGMENT

We wish to express our appreciation to Miss Alice Sprenger for her patient and efficient technical assistance

#### REFERENCES

- <sup>1</sup> WHARTON JONES T The blood corpuscle considered in its different phases of development in the animal series Phil Trans R Soc. London *x* 82, 1846
- <sup>2</sup> SCHULTZE M Ein heizbarer Objecttisch und seine Anwendung bei der Untersuchung des Blutes Arch f mikr Anat *x* 1 1865
- <sup>3</sup> CUNNINGHAM, R S AND TOMPKINS, E H The supravitral method of studying blood cells In Hal Downey Handbook of Hematology Paul B Hoeber 1938 553
- <sup>4</sup> EHRLICH P Über die spezifischen Granulationen der Blutes Arch f Anat u Physiol Phys Abt 1879 (Verhandl d Berl Phys Gesellsch 16/5) 571
- <sup>5</sup> SCHWARZ, E Die Lehre von der allgemeinen und örtlichen Eosinophilie Ergebn d allg Path u path Anat *17*, part 1, 137 1914
- <sup>6</sup> BETHELL, F H STURGIS C C RUNDLES, R W AND MEYERS, M C Progress in internal medicine Blood A Review of the Recent Literature Arch Int Med *77* 196 1946
- <sup>7</sup> Ehrlich P and Lazarus A Die Anaemie (in Nothnagel's Spec. Pathologie) 1st edition 1898
- <sup>8</sup> ASCOLI M Über die Entstehung der eosinophilen Leukocyten Fol haematol *1* 683 1904
- <sup>9</sup> PAPPENHEIM A Zur Frage der Entstehung der eosinophilen Leukozyten Fol haematol *2* 166 1905
- <sup>10</sup> WEIDENREICH F Zur Frage nach der Entstehung der eosinophilen Lenkozyten Fol haematol *2* 163 1905
- <sup>11</sup> MAXIMOW A Untersuchungen über Blut und Bindegewebe I Die frühesten Entwicklungsstadien der Blut und Bindegewebszellen beim Säugetier-embryo bis zum Anfang der Blutbildung in der Leber Arch f mikr Anat *73* 444, 1909
- <sup>12</sup> DOWNEY H The origin and development of eosinophil leucocytes and of hematogenous mast cells in the bone marrow of adult guinea pigs Fol haematol Arch *19* 148 1915
- <sup>13</sup> RINDGEN A R The origin of the eosinophil leucocytes of mammals Fol haematol Arch *17* 10, 1921
- <sup>14</sup> BIGGART J H Some observations on the eosinophile cell J Path & Bact. *35* 799 1932
- <sup>15</sup> OPIE E L The occurrence of cells with eosinophile granulation and their relation to nutrition Am J M Sc *127* 217 1904 An experimental study of the relation of cells with eosinophile granulation to infection with an animal parasite (*Trichina spiralis*) Am J M Sc *127* 477 1904
- <sup>16</sup> SCHLECHT H AND SCHWENKER G Über lokale Eosinophilie in den Bronchien und in der Lunge anaphylaktischer Meerschweinchen Arch f exper Path u Pharm *68* 163 1912. Über die Beziehungen der Eosinophilie zur Anaphylaxie Deutsche Arch Klin Med *108* 405 1912.
- <sup>17</sup> WEINBERG M AND SEGUIN P Recherches biologiques sur l'eosinophilie Ann Inst Pasteur *21* 470, 1914. Recherches biologiques sur l'eosinophilie II Propriétés phagocytaires et absorption d. produits vermineux Ann Inst Pasteur *29* 323 1915
- <sup>18</sup> HANNA E Pathnlogische und biologische Untersuchungen über die Eosinophil Zellen und die Eosinophilie. Virchow's Arch f path Anat *233* 11 1921
- <sup>19</sup> COOKE R A Infective Asthma Indication of its Allergic Nature Am J M Sc *183* 309 1931
- <sup>20</sup> RINGOEN A R Eosinophile Leucocytes and Eosinophilia In Hal Downey Handbook of Hematology Paul B Hoeber 1938 179
- <sup>21</sup> HAJOS, K Beiträge zur Eosinophilie Frage II Mitteilung Die Verteilung der eosinophilen Zellen

- nach Proteininjektionen und in anaphylaktisierten Meerschweinchen *Zeitschr f d ges exp Med* 59 353 1928
- III Mitteilung Über den Zusammenhang zwischen der Bluteosinophilie und der Immunkörperbildung *Zeitschr f d ges exp Med* 59 389 1928
- AND MARCON R. Beiträge zur Eosinophiliefrage IV Mitteilung Die Desensibilisierungseosinophilie *Zeitschr f d ges exp Med* 68 767 1929
- <sup>1</sup> CAMPBELL, A C P, DRENAN A M AND RETTIE T. The relationship of the eosinophile leucocyte to allergy and anaphylaxis *J Path & Bact* 40 537 1935
- <sup>21</sup> CAMPBELL, D H. Experimental eosinophilia with Keratin from *Ascaris suum* and other sources *J Infect Dis* 51 250 1942 Relationship of the eosinophil response to factors involved in anaphylaxis *J Infect Dis* 52 42 1943
- <sup>22</sup> LEIENBERGER CARL. Die Blutmorphologie der Laboratoriumstiere Leipzig Johann Ambrosius Barth 1927
- <sup>23</sup> SCHLECHT H. Über experimentielle Eosinophilie nach parenteraler Zufuhr artfremden Eiweisses und über die Beziehungen der Eosinophilie zur Anaphylaxie *Arch f exp r Path u Pharm* 67 137, 1912
- <sup>24</sup> COHEN M B AND FRIEDMAN H J. Histamine Azoprotein in the Treatment of Allergy *J Allergy*, 18 7 1947
- <sup>25</sup> PIQUET, C VON AND SCHICK B. Die Serumkrankheit Wien 1925
- <sup>26</sup> HEIDELBERGER M, TREFFERS H P AND FREUND J. Protein antiprotein reactions in horse sera *Fed Proc* 2 178 1942
- <sup>27</sup> ZAPPERT J. Eine Methode zur Zählung der eosinophilen Zellen im frischen Blut *Zentralbl klin Med* 1892 386
- <sup>28</sup> DISCOMBE G. Criteria of Eosinophilia *Lancet* 250 195 1946
- <sup>29</sup> RANDOLPH TH G. Blood Studies in Allergy I The Direct Counting Chamber Determination of Eosinophils by Propylene Glycol Aqueous Stains *J Allergy* 15 89 1944
- <sup>30</sup> RICH A R. The Role of Hypersensitivity to the Pathogenesis of Rheumatic Fever and Periarthritis Nodosa. Twenty First Lewis Lion McArthur Lecture of the Frank Billings Foundation Inst of Med Chicago Feb 23 1945
- <sup>31</sup> FEINBERG S M. Histamine and Antihistaminic Agents Their Experimental and Therapeutic Status Report to the Council on Pharmacy and Chemistry *J A M A* 132 702, 1946
- <sup>32</sup> LOEW, E R. Pharmacology of Antihistaminic Compounds *Physiotherapy Rev* 27 542 1947
- <sup>33</sup> ACHENBACH P AND LOEW E R. Antagonism of histamine with synthetic compounds which exert marked epinephrine blocking effects *Fed Proc* 6 304 1947
- <sup>34</sup> MAYER R L. Antihistaminic Substances With Special Reference to Pyribenzamine *J Allergy* 17 153 1946
- <sup>35</sup> CODE, C F. The Mechanism of Anaphylactic and Allergic Reactions An Evaluation of the Role of Histamine in Their Production *Ann of Allergy* 2 457 1944
- <sup>36</sup> An Evaluation of Benadryl Pyribenzamine and other so-called Antihistaminic Drugs in the Treatment of Allergy *J Pediatr* 30 583, 1947
- <sup>37</sup> SCHWENKER G AND SCHLECHT H. Über den Einfluss sympathiko- und autonomo-troper Substanzen auf die eosinophilen Zellen *Zeitschr klin Med* 75 77 1912
- <sup>38</sup> NEUKER, E VON, Haematologische Mitteilungen I *Wien klin Wchnschr* 41 64 1892.
- <sup>39</sup> BERTELLI G, FALTA W AND SCHWENKER O. Über die Wechselbeziehung der Drüsen mit innerer Sekretion III Über Chemotaxis *Zeitschr klin Med* 71 23 1910
- <sup>40</sup> CAMP W J R. The effect of drugs on the number of circulating white blood cells *J Lab & Clin Med* 13 206 1927
- <sup>41</sup> DOBREFF M, DOITSCHINOFF L S AND MARINOFF B. Über das Verhalten der Bluteosinophilie bei der Adrenalinleukocytose (Untersuchungen am klinischen Material) *Klin Wchnschr* 13 1536 1934.
- <sup>42</sup> VAUGHAN, W T. Food Allergens III The leucopenic index preliminary report *J Allergy* 5 601 1934 The leucopenic index as a diagnostic method in the study of food allergy with a discussion of its reliability *J Lab & Clin Med* 21 1278 1936
- <sup>43</sup> DRAOSTEDT C A. Anaphylaxis *Physiotherapy Rev* 21 563 1945
- <sup>44</sup> ROCHA e SILVA M. Anaphylaxis in the Dog *Lect deliv at Chicago Med School* August 15, 1946

- <sup>40</sup> BEST, CH H AND TAYLOR, N B *The Physiological Basis of Medical Practice* ed 4 page 253 Baltimore, Williams & Wilkins, 1945
- <sup>41</sup> HALPERN, B N Experimental Research on a New Series of Chemical Substances with Powerful Antihistaminic Activity The Thiophenylamine Derivatives *J Allergy* 18 263 1947
- <sup>42</sup> INGRAHAM, E. S AND WORTMAN, W B Chemotropism of human eosinophil polymorphonuclear leukocytes *Arch Path* 48 318, 1939
- <sup>43</sup> GERLACH, W Studien über hyperergische Entzündung Virchow's Arch f path Anat 247 294, 1923
- <sup>44</sup> KLINE, B S, COREN, M B AND RUDOLPH, J A Histologic changes in allergic and non allergic wheals *J Allergy* 3 532, 1932
- <sup>45</sup> JADASSON, W Eosinophile und urticarielle Quaddel *Arch f Derm u Syph* 166 458, 1932
- <sup>46</sup> KNOTT, F Q AND PEARSON, R. G B Eosinophilia in Allergic Conditions *Guy's Hosp Rep* 14 230, 1934
- <sup>47</sup> OSGOOD, E E Marrow Cultures In Symposium on the blood and blood forming organs Madison, University of Wisconsin, 1939
- <sup>48</sup> KIRK R C The Causes of Eosinophilia *Internat Clin I series* 5 219, 1942

# A FACTOR IN SERUM WHICH ACCELERATES THE CONVERSION OF PROTHROMBIN TO THROMBIN I ITS DETERMINATION AND SOME PHYSIOLOGIC AND BIOCHEMICAL PROPERTIES\*

By A. DE VRIES,† M.D., B. ALEXANDER, M.D., AND R. GOLDSTEIN, M.D.

With the technical assistance of E. ADDELSON, A.B. AND E. PROMISEL

## INTRODUCTION

IN 1945, Nolf<sup>1</sup> described a serum constituent, distinct from thrombin, which was capable of furthering coagulation. More recently, Ware et al.<sup>2-4</sup> reported a substance, designated serum Ac globulin, which activates the conversion of prothrombin to thrombin by thromboplastin. Independently, Owren<sup>5</sup> discovered a new clotting factor, Factor VI, which arises during coagulation, speeds the evolution of thrombin, and catalyzes its own formation. These observations are of fundamental importance in our knowledge of blood clotting since they help explain the autocatalytic process underlying the evolution of thrombin.

We observed that the admixture of serum to plasma accelerates the conversion of prothrombin to thrombin following the addition of thromboplastin plus calcium. This report presents data regarding some physiologic and biochemical properties of the agent (serum prothrombin conversion accelerator) responsible for this effect, together with a method for its estimation. A study of its role in blood coagulation and in the pathogenesis of certain hemorrhagic disorders is reserved for subsequent communications.<sup>6</sup>

## METHODS

*General Considerations.* The determination of plasma prothrombin by the one stage technic<sup>7</sup> has two disadvantages: (a) the prothrombin time is considerably influenced by both the concentration of prothrombin and certain nonprothrombin substances<sup>8-12</sup>, (b) above 50 per cent (of normal) prothrombin concentration the decrement in prothrombin time with significant increment in prothrombin is so small as to be almost within the limits of error of measurement. To obviate these disadvantages, oxalated plasma was suitably diluted with normal plasma rendered essentially free of prothrombin by prior adsorption with barium sulfate according to the technique of Rosenfield and Tuft.<sup>13</sup> Plasma so treated† contains adequate amounts of nonprothrombin substances which affect the prothrombin time.<sup>14, 15</sup>

This technic is applicable also to the determination of the prothrombin activity of serum. Since however serum contains a factor which accelerates the conversion of prothrombin to thrombin, it is a priori evident that the prothrombin times may reflect the concentration of both prothrombin and the accelerator. It would therefore be incorrect to derive serum prothrombin concentrations from prothrombin times. Accordingly we have interpreted the latter in terms of prothrombin activities.

*Plasma Prothrombin.* Plasma prothrombin was determined by the method of Rosenfield and Tuft.<sup>13</sup> 0.1 cc. of oxalated plasma (1 volume of 0.1 M sodium oxalate solution to 9 volumes of blood) was mixed

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From the Medical Research Laboratory, Beth Israel Hospital, and the Department of Medicine, Harvard Medical School, Boston, Massachusetts.

\* Supported by a grant from the Commonwealth Fund.

† Aided by a fellowship from the Rothschild Hadassah University Hospital, Jerusalem.

‡ Referred to as BaSO<sub>4</sub> plasma throughout this paper.

with 0.9 cc of fresh oxalated  $\text{BaSO}_4$  plasma. The latter was prepared in the following manner: barium sulfate (C.P.) was added to plasma (100 mg per cc). The mixture was shaken, incubated at 37°C for ten minutes, with repeated shaking and centrifuged at 3000 r.p.m. for thirty minutes. The supernatant was kept at 0-4°C and was used within two hours after its preparation as the diluent for the test plasma.

Thromboplastin extracts were prepared every two weeks from commercial (Difco) thromboplastin. 0.1 cc aliquots were pipetted into prothrombin time tubes which were then stored at -23°C. Under such conditions the potency remains unchanged for at least two weeks.<sup>10</sup> Immediately before use the tubes containing the frozen thromboplastin were thawed for 10 minutes at 37°C, 0.1 cc of the plasma mixture was then added followed by 0.1 cc of 0.02 M  $\text{CaCl}_2$  solution and the time of clotting observed while the mixture was constantly stirred with a wire loop. All determinations were done at least in duplicate.

A curve relating prothrombin concentration with prothrombin time was derived from determinations on normal plasma serially diluted with increasing amounts of  $\text{BaSO}_4$  plasma. Plasma prothrombin activity was computed from this curve after correction for dilution.

*Serum Prothrombin Activity.* Serum prothrombin activity was determined in the same manner. Unless otherwise indicated, venous blood was allowed to clot spontaneously at room temperature. After standing for one hour the clot was spun and the serum separated. To 9 volumes of serum was added one volume of 0.1 M sodium oxalate solution; the mixture was incubated for 30 minutes at 37°C, and thereafter kept in the refrigerator until used (within three hours). Since the prothrombin activity of normal serum is low, the proportion of serum to  $\text{BaSO}_4$  plasma in the mixture to be tested was 3 to 7. Such proportions assure adequate amounts of nonprothrombin factors which affect the prothrombin time.<sup>11</sup>

*Serum Prothrombin Conversion Accelerator (SPCA).* 0.05 cc of oxalated plasma were mixed with 0.9 cc of  $\text{BaSO}_4$  plasma, 0.05 cc of oxalated serum were then added and the prothrombin time determined on 0.1 cc of the mixture. The SPCA was calculated by subtracting the algebraic sum of the individual prothrombin activities of the serum and plasma components from the observed prothrombin activity of the mixture, the value thus obtained divided by the algebraic sum gives the percentage enhancement of prothrombic activity. For example: Assume the plasma to contain 100 per cent (of normal) prothrombin activity and serum 10 per cent. A mixture of equal volumes of both should show 55 per cent activity. If the observed value is 120 per cent, the SPCA is

$$\frac{120 - 55}{55} \times 100 = 118 \text{ per cent enhancement}$$

### PHYSIOLOGIC PROPERTIES

*Demonstration of SPCA.* The addition of oxalated serum to oxalated plasma diluted with  $\text{BaSO}_4$  plasma shortens the prothrombin time markedly (table 1).

*Accuracy of SPCA Assay.* Aliquots of serum from a blood sample from one subject were mixed with aliquots of plasma and  $\text{BaSO}_4$  plasma obtained from a second subject. The SPCA's of 9 of these aliquot mixtures ranged from 138 to 168 per cent with a mean of 149 (S.D. 8.6). Ten observations made with the same serum as above but mixed this time with plasma and  $\text{BaSO}_4$  plasma from a third individual ranged from 118 to 162 with a mean of 139 (S.D. 12.3). The standard deviation of all 19 observations was 18.7.

*Is SPCA Identical with Thrombin?* The question arises whether SPCA activity is referable to serum thrombin which is demonstrable in serum shortly after coagulation. Incubating serum for one-half hour does not decrease SPCA (table 2), although thrombic activity is inactivated by serum antithrombin. To prove that this serum could inactivate considerable amounts of thrombin, 125 units (Parke Davis, Topical Thrombin) were added to 1.0 cc. A drop of the mixture added immediately to 0.5 cc of plasma resulted in instantaneous clotting. The same mixture, incubated for one-half hour at 37°C, gave no clot in 1 hour when added to plasma, and con

tained less than 1.25 units of thrombin when its coagulating effect was compared with that of a standard thrombin preparation under standard conditions.<sup>5</sup>

The binding of thrombin by serum antithrombin is an equilibrium reaction in which a small amount of thrombin may remain free.<sup>17</sup> Conceivably, SPCA activity may be due to a trace of thrombin which might not otherwise be demonstrable. This was excluded by the following experiment (table 3). Small amounts of

TABLE 1.—*Demonstration of SPCA*

Mixture (cc)				Pro T sec	Prothr activity*			SPCA †
Oxal Plas	Ser	Sal	BaSO <sub>4</sub> pla		a	b	c	
					Plas Sal Mixt	Ser	Plas ser Mixt	
					%	%	%	%
0.05	0	0.05	0.90	41.4	43			
0	0.40	0	0.60	55		7.5		
0.05	0.05	0	0.90	24.6			94	101

\* Corrected for dilution with BaSO<sub>4</sub> plasma

$$\dagger c - \left( a + \frac{b}{2} \right) \\ a + \frac{b}{2}$$

TABLE 2.—*Comparative SPCA Activities of Fresh and Incubated Serum*

Mixture (cc)					Pro T sec	Prothr activity†			SPCA
Oxal plas	Serum		Sal	BaSO <sub>4</sub> plas		a	b	c	
	Non incub	Incub				Plas sal mixt	Ser	Plas ser mixt	
						%	%	%	%
o 05			o 05	o 90	42	41			
o 05†	o 05			o 90	23 4			100	117
o 05		o 05		o 90	23 2			102	122
	o 10			o 90	>180		<10		

\* Incubated 30 minutes at 37°C.

† This experiment was run immediately after the addition of the serum to the plasma barium sulfated plasma mixture in order to obviate spontaneous clotting resulting from thrombin in the serum.

‡ Corrected for dilution with BaSO<sub>4</sub> plasma

thrombin added to plasma-barium sulfated plasma mixtures had a negligible effect on the prothrombin time although the added thrombin induced clotting after a latent period. The addition of serum *which had a large SPCA activity*, on the other hand, did not result in coagulation in the same interval.

*Does SPCA Activity Affect the Reaction between Fibrinogen and Thrombin?* The prothrombin time measures the speed of both prothrombin conversion to thrombin and the reaction of thrombin with fibrinogen. Theoretically, SPCA might act by accelerating the second of these reactions. Mixtures of plasma, serum and BaSO<sub>4</sub>

plasma were prepared in the usual manner and SPCA determined. To 0.4 cc of the mixtures were added 0.4 cc of a veronal sodium chloride buffer (pH 7.38), prepared according to Owren,<sup>5</sup> and 0.2 cc of thrombin solution (Parke-Davis Topical Thrombin) containing 2.5 or 5 units per cc (in Owren's buffer). The data (table 4) show that SPCA does not affect the speed of fibrinogen transformation into fibrin.

TABLE 3—Comparative Effects of Thrombin and Serum on Clotting of Plasma and on the Prothrombin Time of Plasma

Mixture (cc )						Pro T sec	Prothr activity				SPCA	Clot in mixt	
Oxal plas	Oxal ser	Sal	Thrombin sol Units per cc		BaSO <sub>4</sub> plas		a Plas sal mixt	b Ser	c. Plas. ser mixt.	c' Plas. throm mixt		Time	+ -
			1.25	0.63									
0 05		0 05			0 90	40 1	45						
	0 30				0 70	55		10					
0 05	0 05				0 90	20 3			125		150	30	-
0 05			0 05		0 90	34 9				51	13	25	+
0 05				0 05	0 90	34 1				50	12	30	-

\* Corrected for dilution with BaSO<sub>4</sub> plasma.

TABLE 4—The Effect of Serum on the Reactivity of Plasma Fibrinogen to Thrombin under the Conditions of the SPCA Test

Mixture (cc)				Pro T sec	Prothr activity†			SPCA	Clotting time (sec onds) following addition of thrombin	
Oxal plas	Sal	Oxal ser	BaSO <sub>4</sub> plas.		a Plas sal mixt	b Ser	c Plas ser mixt.		1 unit	½ unit
0.05	0.05		0.90	39.9	47				24.3	46.2
		0.30	0.70	90		4				
0.05		0.05	0.90	26.2			103	110	24	45.9

\* To 0.4 cc. mixture was added 0.4 buffer pH 7.38 (Owren<sup>5</sup>) and 0.2 cc of a thrombin solution of 5 units or 2½ units per cc. Clotting time determined at 37°C.

† Corrected for dilution with BaSO<sub>4</sub> plasma.

*Quantitative Relationship between SPCA Effect and Plasma-Serum Ratio in Mixture*  
SPCA activity was determined on mixtures in which the concentrations of serum and plasma were varied. With 0.02 to 0.30 cc of plasma were mixed 1.30 cc of BaSO<sub>4</sub> plasma. To each of the mixtures, 0.05 cc of serum were added, the volumes were adjusted to 2.00 cc with physiologic saline, and the prothrombin times measured. In other experiments the plasma was kept constant and the serum was varied, viz. To a mixture of 0.05 cc plasma and 1.30 cc of barium sulfated plasma were added 0.02 to 0.50 cc of serum, and saline to a final volume of 2.00 cc. As the serum concentration increases, the velocity of conversion of plasma prothrombin

to thrombin increases (figs. 1, 3). Maximal SPCA activity is reached when the serum concentration is approximately tenfold that of plasma. The effect of maintaining the serum constant and varying the plasma concentration is shown in figures 2 and 3.

*Is SPCA Activity Due to Thromboplastin?* Very recently, Chargaff<sup>18</sup> reported that thromboplastin is not consumed during blood coagulation. This contradicts the claim of Mertz et al.<sup>19</sup> It has furthermore been shown that platelet extracts can accelerate the prothrombin time of fresh or stored plasma.<sup>20</sup> The possibility that

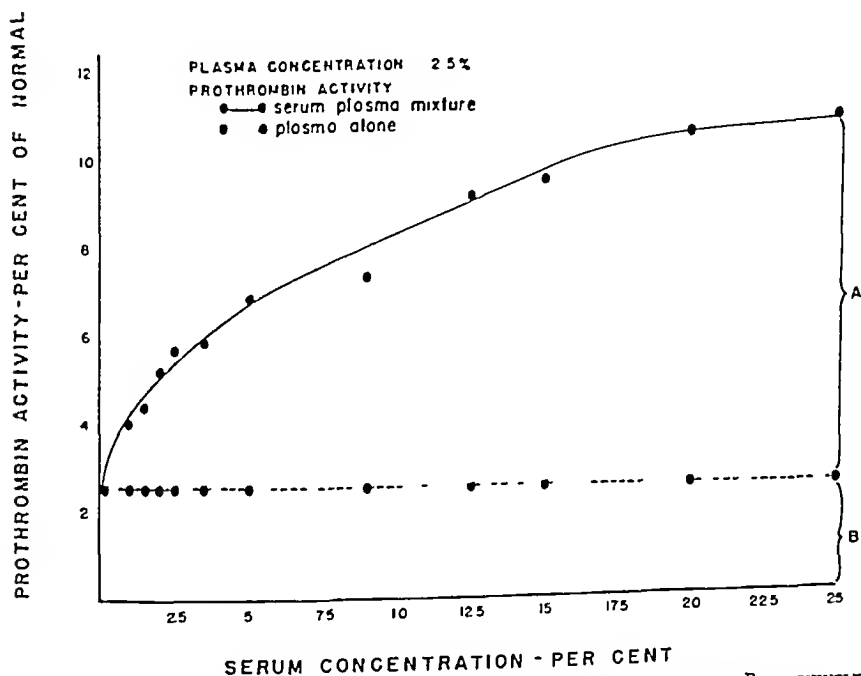


FIG. 1—ENHANCEMENT OF PLASMA PROTHROMBIN ACTIVITY INDUCED BY ADMIXTURE OF PROGRESSIVELY INCREASING AMOUNTS OF PROTHROMBIN FREE SERUM  $\frac{A}{B} \times 100 = \text{SPCA Activity}$

the clot promoting effect of serum might be due to unconsumed thromboplastin required investigation.

The addition of thromboplastin extracts (0.1 cc. of Difco prepared as for prothrombin determination) to 2.0 cc. of oxalated serum resulted in no enhancement of its SPCA activity. Furthermore, thromboplastin (0.05 cc.) added to a mixture of 0.05 cc. plasma and 0.9 cc. of  $\text{BaSO}_4$  plasma did not alter the prothrombin time. Platelet extracts obtained with saline, distilled  $\text{H}_2\text{O}$  or a solution of saponin also gave negative results.

*Relation Between SPCA and Labile Factor* As plasma is stored, its prothrombin activity decreases although the concentration of prothrombin remains unchanged.<sup>21</sup>



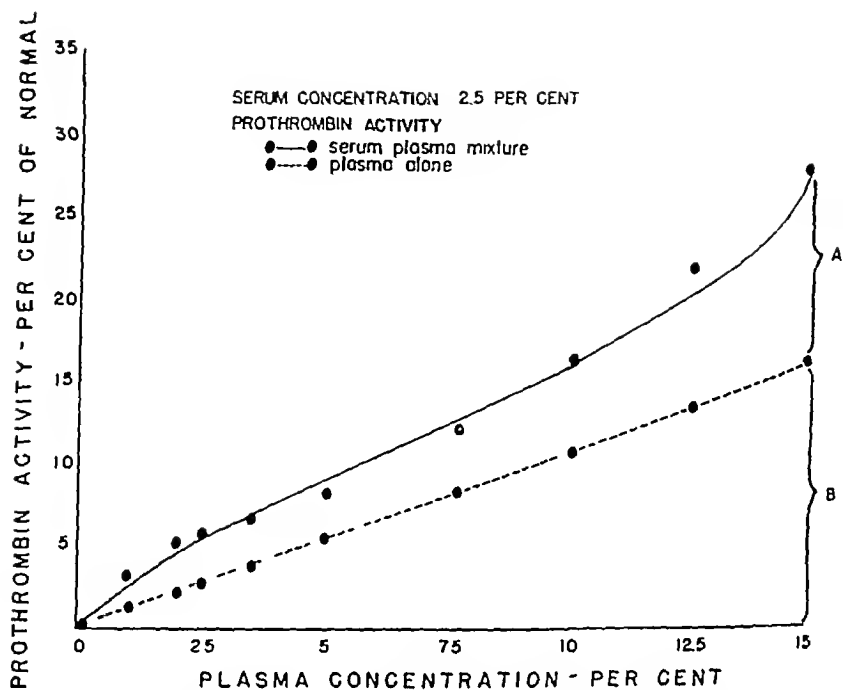


FIG. 2.—PLASMA PROTHROMBIN ACTIVITY IN PLASMA-SERUM  $\text{BaSO}_4$  PLASMA IN WHICH SERUM CONCENTRATION IS FIXED AND PLASMA CONCENTRATION IS VARIED  $\frac{A}{B} \times 100 = \text{SPCA Activity}$

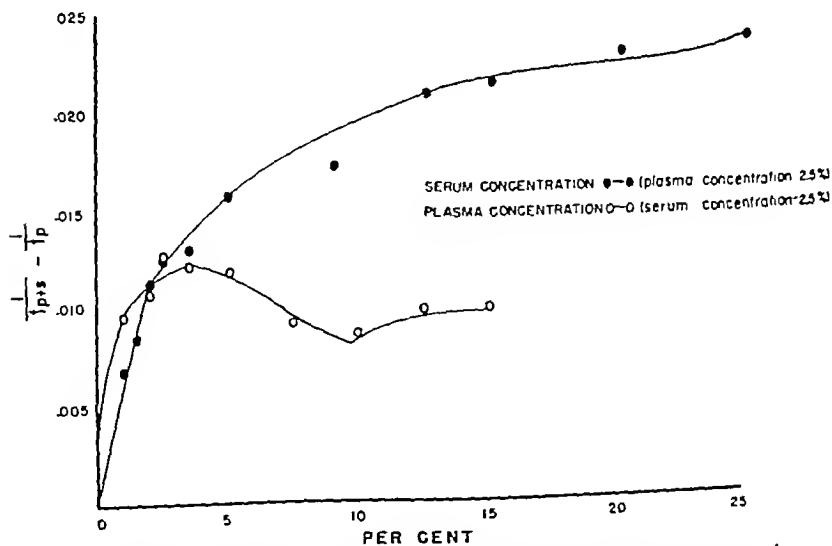


FIG. 3.—EFFECT OF SERUM ON VELOCITY OF PROTHROMBIN CONVERSION TO THROMBIN  $t_p + s$  is the prothrombin time of the plasma serum mixture  $t_p$  is the prothrombin time of the plasma

This is due to deterioration of a labile factor.<sup>8</sup> The addition of prothrombin free fresh plasma to stored plasma restores the prothrombin time to normal. According to Mann et al.<sup>10</sup> and Munro and Munro,<sup>11</sup> serum also can lower the prothrombin time of stored plasma. This has also been confirmed by us. The question whether this restorative ability of serum is referable to its labile factor or to SPCA required elucidation.

When normal human blood is added immediately to thromboplastin extract (9.0 cc blood to 1.0 cc Difco thromboplastin extract), its serum often contains much more SPCA activity than the serum of blood allowed to clot spontaneously.<sup>6</sup> An experiment was done in which a serum, thus prepared, exhibited marked SPCA activity, although its restorative effect on the prothrombin time of stored plasma was negligible (table 5). It should be noted, however, that serum obtained from blood drawn into thromboplastin is not always devoid of labile factor.

TABLE 5—Comparison of SPCA Activity of Serum with its Ability to Reactivate the Prothrombin of Stored Plasma

Oxal serum	Serum prothr activity	SPCA activity on fresh plasma	Restorative effect of serum on stored plasma Prothrombin time	
			Stored plasma	Stored plasma plus serum (1:1)
	%	%	sec	sec
From blood clotted with thromboplastin supplement	0	300	87	52
From blood clotted spontaneously				
Fresh	4	121	87	18.4†
Incub 4 hrs at 37°C	<3	100	80	44.2
Kept 26 hrs at room temp	1	75	89	69

\* Oxalated human plasma aged 32 days at 4°C

† This value is comparable to the prothrombin time (17.8 sec) obtained on a 1:1 mixture of this stored plasma with fresh plasma.

Labile factor deteriorates more rapidly at 37°C than at refrigerator temperature.<sup>22, 13</sup> In serum aged at body or at room temperature labile factor deteriorates much more rapidly than SPCA (table 5). Additional experiments on the lability of SPCA and other biochemical properties will be presented later.

*Effect of Serum on the Prothrombin Conversion Rate of Stored Plasma with and without Supplements of Labile Factor.* Oxalated serum from blood drawn into thromboplastin was kept at room temperature for twenty-six hours. It was free of prothrombin and rich in SPCA. A mixture of equal parts of this serum with stored plasma whose prothrombin time was 90 seconds (indicating 12 per cent of normal prothrombin activity) gave a prothrombin time of 116 seconds (indicating 10 per cent prothrombin activity). When, however, the mixture was diluted 1 to 9 with BaSO<sub>4</sub> plasma (rich in labile factor), the prothrombin activity of the serum-plasma mixture was almost twice that of the plasma alone similarly diluted with BaSO<sub>4</sub> plasma (table 6). It appears that SPCA needs, for its activity to become manifest, a factor present in fresh plasma, whole or barium sulfated, and absent in stored plasma.

*SPCA Activity in the Presence of Heparin* Heparin added to serum-plasma mixtures in concentrations capable of lengthening the prothrombin time of plasma alone did not abolish SPCA activity (table 7)

TABLE 6—*SPCA Activity at Low and Normal Concentration of Labile Factor*

Mixture (Parts)				Prothrombin	
Stored plas	Ser †	Sal	Fresh BaSO <sub>4</sub> plas	Time	Activity per cent of normal
				sec.	
I				90	12
	3		7	>3 min.	0
I	I			116	10
I		I	18	29.4	80†
I	I		18	23.4	155
			I	>3 min.	0

\* Corrected for dilution with BaSO<sub>4</sub> plasma.

† This high prothrombin activity of stored plasma diluted with BaSO<sub>4</sub> plasma has been reported in a previous communication<sup>12</sup> Its explanation is still obscure

‡ This serum was kept at room temperature for 26 hours

TABLE 7—*Acceleration of Prothrombin Activity by Serum in the Presence of Heparin*

Mixture				Heparin† solution		Prothrombin	
Oxal plas	BaSO <sub>4</sub> plas	Sal	Oxal ser			Time	Activity per cent of normal
cc	cc	cc	cc	cc.	units per cc	sec	
0.05	0.85	0.10				47.7	25.4
0.05	0.85	0.05	0.05			28.7	48
	0.70		0.30			39.1	15
0.05	0.85	0.05		0.05	100	>3 min	<6.5
0.05	0.85		0.05	0.05	100	>3 min	<6.5
0.05	0.85	0.05		0.05	10	59.3	18.7
0.05	0.85		0.05	0.05	10	35.6	32.6
0.05	0.85	0.05		0.05	5	58.1	20.0
0.05	0.85		0.05	0.05	5	33.4	35.3
0.05	0.85	0.05		0.05	2.5	50.8	21.3
0.05	0.85		0.05	0.05	2.5	32.8	36.6

\* Corrected for dilution with BaSO<sub>4</sub> plasma.

† Heparin, Upjohn (1000 units per cc) This was diluted with physiological saline

### *Biochemical Properties of SPCA*

*Stability* The stability of SPCA in oxalated serum subjected to different temperatures for varying intervals of time is shown in table 8

*Precipitation by CO<sub>2</sub>* SPCA is not precipitable by CO<sub>2</sub> from oxalated serum diluted 1 to 10 with distilled water. All the activity is demonstrable in the supernatant.

*Adsorption by BaSO<sub>4</sub> or Seitz Filter* Serum was treated with BaSO<sub>4</sub> according to the procedure for adsorbing prothrombin. Its SPCA was removed incompletely.

TABLE 8—Stability of SPCA Activity at Various Temperatures  
pH 7.8

Treatment of oxal. ser.		Per cent of orig. SPCA activity remaining
Temp	Interval	
C		
4	24 hrs	88
4	11 days	71
25	26 hrs	62-83
37	4 hrs	80-83
45	6 min	100
56	2½ min	10
56	30 min	0

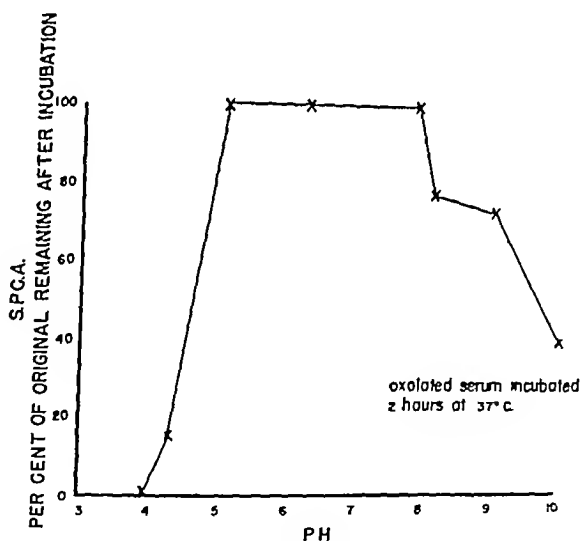


FIG. 4—SPCA SENSITIVITY TO VARYING pH's

and erratically. If, however, the serum was oxalated prior to such treatment, the SPCA was removed quantitatively.

Oxalated serum (25-40 cc) filtered through a Seitz pad (Hercules Type S, size 13, diam 3.6 cm) was devoid of both SPCA and prothrombin activity.

*Sensitivity to Various Hydrogen Ion Concentrations* Oxalated serum was brought to various pH's by the addition of small amounts of acetic acid or sodium hydroxide while the mixture was constantly agitated. After being kept at these pH's for two

hours at room temperature the sera were readjusted to a pH of 7.7 to 8.3 and their SPCA activities immediately determined (fig. 4)

*Behavior of SPCA to Dialysis* The SPCA activity of oxalated serum dialyzed for 25 hours at 9°C in a cellophane bag against oxalated physiologic saline solution was essentially unchanged

*Heterogeneity of SPCA* Dog serum (prothrombin free) can accelerate prothrombin conversion in either dog or human plasma, and its SPCA activity is greater than that of human serum. It should be emphasized that these results are obtained whether the diluent of the plasma-serum mixture is dog, or human, BaSO<sub>4</sub> plasma. Rabbit serum (prothrombin free) also showed excellent SPCA activity on human plasma prothrombin.

### DISCUSSION

Recently various investigators<sup>4, 5</sup> have described clot promoting substances, distinct from thromboplastin and thrombin, which arise *de novo* during blood coagulation. The serum Ac-globulin of Ware et al.<sup>4</sup> evolves from plasma Ac-globulin which itself is relatively inert. Conversion of the plasma component into the serum moiety is said to be affected by extremely small amounts of thrombin. Owen<sup>5</sup> Factor VI (prothrombinase) arises in a mixture of thromboplastin, ionized calcium, prothrombin and Factor V.

Both serum Ac-globulin and Factor VI catalyze the conversion of prothrombin to thrombin. To assay the former, an adaptation of the two stage prothrombin method is used.<sup>3</sup> Factor VI is measured by the amount of additional thrombin evolved from prothrombin in oxalated plasma following addition of the above described reaction mixture in which Factor VI has developed. For both procedures purified prothrombin is required.

In the one stage prothrombin method the clotting time reflects, *inter alia*, the concentration of prothrombin and the velocity of its conversion to thrombin. Modification of this method by dilution with BaSO<sub>4</sub> plasma lends itself well to the study of serum factors which affect the speed of this reaction. This technic dispenses with purified components or isolated systems, and is no more complicated than the routine one stage dilution technique for the determination of plasma prothrombin.

The effect of serum in enhancing the prothrombin activity of plasma is not referable to thrombin, thromboplastin or substances obtainable from platelets. The factor acts apparently on the velocity of prothrombin conversion to thrombin. Accordingly it was designated serum prothrombin conversion accelerator (SPCA), pending further investigation regarding its possible identity with other substances having similar physiologic properties.<sup>1, 2</sup>

SPCA is distinct from the labile factor which also influences the velocity of prothrombin conversion. The ability of serum to reactivate the prothrombin activity of stored plasma is not due to SPCA. This substantiates the conclusions of other investigators that serum contains labile factor.<sup>10, 11</sup> That SPCA has negligible effect on the velocity of thrombin evolution in stored plasma in

which labile factor has largely deteriorated indicates that the latter is necessary for SPCA activity to be fully manifest.

While insufficient data are available to establish the identity or nonidentity of SPCA with Factor VI and serum Ac-globulin, it may be worth-while to compare certain of their properties. Ac-globulin is relatively stable in plasma and serum, is almost quantitatively precipitated from aqueous solution at pH 5.4, is not adsorbed by barium carbonate, and seems to be sensitive to alkaline pH's.<sup>1,2,3</sup> SPCA is similarly stable and sensitive to alkaline pH's. It is, however, not precipitated from diluted serum by CO<sub>2</sub> (pH 5.8) and is adsorbable by barium sulfate from oxalated serum. If SPCA is identical with Ac-globulin the factor is more stable in serum than in purified form since in the latter state about one-half is destroyed at 37°C within 30 minutes,<sup>4</sup> whereas only about 20 per cent of SPCA is destroyed in serum at this temperature within four hours.

There is one important distinction between SPCA and serum Ac-globulin. The latter arises from the action of thrombin on plasma Ac-globulin which, as an accelerator of prothrombin conversion, is relatively inert.<sup>2,3</sup> Very potent preparations of serum Ac-globulin were obtained from plasma to which highly purified thrombin had been added. In contrast, we were unable to produce SPCA in plasma by adding thrombin. In subsequent publications,<sup>6</sup> furthermore, data will be presented indicating that very little, if any, SPCA could be demonstrated in various pathologic states, despite the fact that thrombin had been formed as indicated by clotting as well as by substantial differences between the prothrombin content of the plasma and that of its respective serum.

Owren<sup>5</sup> concludes that Factor VI is identical with Fischer's<sup>25</sup> autocatalytic clot promoting agent which arises during coagulation. If this is so, SPCA is not Factor VI, since Fischer states that serum is devoid of this agent.

#### CONCLUSIONS

1. The addition of oxalated serum to oxalated plasma accelerates the conversion of prothrombin to thrombin in the presence of optimal amounts of thromboplastin plus calcium.
2. The serum agent responsible for this effect is distinct from thrombin, thromboplastin or labile factor. We have called it the serum prothrombin conversion accelerator (SPCA).
3. A method for its assay is presented.
4. Some of its physiologic and biochemical properties are described.

#### REFERENCES

- <sup>1</sup> NOLF, P. *De hémophile Schweiz. Med. Wchnschr.* 75: 78 (1945); 75: 110 (1945).
- <sup>2</sup> WARE, A. G., MURPHY, R. C. AND SEEGER, W. H. The function of Ac globulin in blood clotting. *Science* 106: 618 (1947).
- <sup>3</sup> — AND SEEGER, W. H. Serum accelerator globulin: quantitative determination, purification and properties. *Fed. Proc.* 7: 131 (1948).
- <sup>4</sup> — AND — Plasma accelerator globulin: partial purification, quantitative determination and properties. *J. Biol. Chem.* 172: 699 (1948).
- <sup>5</sup> OWREN, P. A. Coagulation of blood. Investigations on a new clotting factor. *Acta Med. Scand. Supplement* 194: 1 (1947).

- <sup>6</sup> ALEXANDER, B DE VRIES, A., AND GOLDSTEIN, R. Studies on a factor in serum which accelerates the conversion of plasma prothrombin to thrombin II Its evolution, with special reference to the influence of conditions which affect blood coagulation Blood, in press
- , AND — Studies on a factor in serum which accelerates the conversion of prothrombin to thrombin III Its relation to the coagulation defect in thrombocytopenic blood Blood, in press
- , AND — Studies on hemophilia V The coagulation defect in hemophilia with special reference to inadequate prothrombin consumption Blood in press
- <sup>7</sup> QUICK, A J The clinical application of the hippuric acid and the prothrombin tests Am J Clin Path 10 222, 1940
- <sup>8</sup> — Components of the prothrombin complex Am J Phys 151 63 1947
- <sup>9</sup> FANTL, P AND NANCE M H Acceleration of thrombin formation by a plasma component Nature 158 708, 1946
- <sup>10</sup> OWREN, P A Parahemophilia Lancet 1 446, 1947
- <sup>11</sup> WARE, A G GUEST, M M AND SEEGER, W H Plasma accelerator factor and purified prothrombin activation Science 106 41 1947
- <sup>12</sup> LAVERGNE G H, AND LAVERGNE POINDESSAULT, B L Stabilité de la prothrombine dans le sang conservé Comp Rend de Soc de Biol 136 445 1942.
- <sup>13</sup> ALEXANDER B, AND DE VRIES, A. Human prothrombin Quantitative studies on the plasma labile factor and the restorative effects of normal, hypofibrinogenemic, and hemophilic plasma on the prothrombin of stored plasma J Clin Investigation in press
- <sup>14</sup> ROSENFELD, R E, AND TUFT, H S Estimation of prothrombin level from prothrombin time Am J Clin Path 17 405 1947
- <sup>15</sup> CONLEY C L, AND MORSE W I Thromboplastic factors in the estimation of prothrombin concentration Am J M. Sc. 215 158 1948
- <sup>16</sup> BLUMGART, H L FREEDBERG, A S, ZOLL, P M LEWIS H D, AND WESSLER S The effect of dicumarol on the heart in acute experimental coronary occlusion Am Heart J 36 13 1948
- <sup>17</sup> SEEGER, W H AND SMITH H P Antithrombic activity of plasma Quantitative interrelationships Proc Soc Exper Biol & Med 52 159 1943
- <sup>18</sup> CHARGAFF E J Studies on the mechanism of the thromboplastic effect J Biol Chem 173 253 1948
- <sup>19</sup> MERTZ E T SEEGER, W H AND SMITH H P Prothrombin thromboplastin and thrombin Quantitative interrelationships Proc. Soc Exp Biol & Med 42 604 1939
- <sup>20</sup> MANN F D HURN M AND MAGATH T H B Observations on the conversion of prothrombin to thrombin Proc Soc Exp Biol & Med 66 33, 1947
- <sup>21</sup> LOOMIS E C AND SEEGER, W H Is prothrombin a unitary principle or a complex? Am J Phys 148 563, 1947
- <sup>22</sup> MUNRO M P, AND MUNRO F L The reversible inactivation of prothrombin A factor responsible for its partial reactivation Am J Phys 150 409 1947
- <sup>23</sup> QUICK A J On the constitution of prothrombin Am J Phys 140 212, 1943
- <sup>24</sup> MURPHY R C, WARE, A G, AND SEEGER, W H Plasma Ac globulin activity Am J Phys 151 338 1947
- <sup>25</sup> FISCHER, A Blutgerinnung als unbegrenzt übertragbare Kettenreaktion Biochem Ztschr 279 108, 1935

# REFINED LIVER EXTRACT IN TROPICAL MACROCYTIC ANEMIA

By J C PATEL, M D , Ph D , AND Y M BHENDE, M D

## INTRODUCTION

NAPIER<sup>1</sup> and Moore et al<sup>2</sup> wrote a few years ago that therapeutic observations with different liver extracts, material containing the extrinsic (food) and/or the hemopoietic factors, would alone help to elucidate the etiology of tropical or nutritional macrocytic anemia(s). Emery and Hurran<sup>3</sup> advocated dosage requirements, based on units, in the treatment of more complex macrocytic anemias and hoped this might lead to the resolution of the divergent views held by different authors on the relative efficacy of crude and refined liver extracts.

Since the early observations of Wills and her associates<sup>4,5</sup> on the inactivity of refined or pure liver extracts in the treatment of tropical macrocytic anemia (T M A ) a number of workers have reported its effectiveness in varying doses. Napier et al<sup>6</sup> found Anahaemin potent in a few cases of T M A. Foy and Kondi<sup>7</sup> and Fairley<sup>8</sup> treated cases of nutritional macrocytic anemia (N M A ) with larger doses of Anahaemin and found it effective. Trowell,<sup>9</sup> in East Africa, observed that 12 ml of Anahaemin per week was required to produce an optimum response in his 6 cases of T M A. Sundaram<sup>10</sup> treated successfully 13 cases of T M A with 12 ml of refined liver extract. Moore et al<sup>2</sup> used Reticulogen in the treatment of 25 cases of N M A of pellagra with good results.

## MATERIAL AND METHODS

The present paper reports the use of refined liver extracts in a series of 45 cases of T M A as found in Bombay. The refined liver extracts used were Anahaemin (British Drug House) Examen (Glaxo) Reticulogen (Lilly) and Examen New Potency (Glaxo).

The cases presented were studied during the last eight years. Thirty one of the 45 cases were investigated in detail according to the procedure advocated by us<sup>11</sup> the rest have been included in this report because they were cases of severe anemia with (1) clinical history and findings similar to those which were diagnosed as T M A after a thorough investigation (2) a high color index (3) predominant macrocytosis in the peripheral blood smear (4) free hydrochloric acid in the gastric juice with or without histamine stimulation. We may emphasize that though the presence of free hydrochloric acid in the gastric juice is in favor of the diagnosis of T M A (as opposed to Addisonian pernicious anemia) its absence does not negate such a diagnosis.<sup>11,14</sup>

All the patients except one had an initial blood count of less than 3,00,000 red blood cells per cu mm. The patients were kept on the hospital milk diet during their first fortnight's stay and later were on an entirely vegetarian diet consisting of chapatis (home made bread of wheat flour) rice dal (a preparation made from pulses) and green and cooked vegetables. It has been our experience (Patel<sup>12</sup>) that no hematologic improvement occurs in patients kept solely on this diet. Besides this hospital diet these cases of anemia were given an alkaline geotian mixture if the gastric analysis showed normal hydrochloric acid content and an acid mixture if there was hyp acidity or achlorhydria. Iron was not administered. Vitamins of the B complex group were administered by mouth or by injection to some of the later cases in addition to the refined liver extract in question. The efficacy of the liver extract therapy was assessed

From the Singhanee Hindu Hospital Bombay and the Department of Pathology Seth G S Medical College Bombay India



## LIVER EXTRACT IN TROPICAL MACROCYTIC ANEMIA

TABLE 1

Case No	R B C in mill per cu mm		Hb in Gm per 100 ml		C I		M C V cu $\mu$		M C H C %		Vanden Bergh reaction	Sternal Puncture			Liver extract used and its total quantity	Max reticulocytes, % and the day	After treatment			Remarks
												Megaloblasts	Normoblasts	Pro-erythroblasts			R B C in mill	Hb in Gm	Days	
1	1 48		5 53	1 27	135	0 28	2				-ve	7 4	35 6	0 0	Anahaemin 2 ml later 2 ml every week for 4 weeks.	14/7	2 36 3 60	9 51 10 72	12 22	Optimum response to a small dose
2	2 72 2 20		11 41 9 36	1 44 1 52	118 122	0 35 0 36	1				-ve -ve	1 5	16 0	3 0	Anahaemin 2 ml Anahaemin 12 ml in 6 days	6/6 10/6	2 20 3 00	9 36 11 76	10 10	No response to small dose but good response to a larger dose
3	0 92		3 46	1 28	123	0 28	3				-ve	3 2	20 9	7 6	Examen 12 ml in 6 days	30/6	1 70 3 44	6 40 11 24	10 39	Optimum response maintained for 39 days
4	1 88		8 99	1 64	127	0 39	0				-ve	10 2	36 0	8 0	Examen 2 ml	4/5	2 88 2 60	11 41 10 20	22 22	Optimum response but not sustained
5	1 70		6 92	1 44	120	0 33	3				-ve	8 3	36 0	0 0	Examen 14 ml in 6 days.	19/7	2 90 3 02	9 51 10 38	10 20	Optimum response but not sustained
6	1 20		5 01	1 37	122	5 34	0				-ve	2 0	42 72	7 8	Examen 14 ml in 6 days	30/6	2 60 2 80	8 47 8 99	10 32	Optimum response but not sustained
7	1 60 1 92		6 05 8 65	1 28 1 53	125 —	0 30 —	25				Indirect +ve -ve	4 0	21 5	2 0	Examen 20 ml in 8 days Campolon 75 ml in 20 days	—	1 92 3 10	8 65 9 68	10 20	Poor response to Examen and comparatively poor response to Campolon
8	1 46		6 05	1 41	115	0 34	4				Indirect +ve	9 3	35 9	18 3	Reticulogen 4 ml in 10 days Campolon 60 ml in 12 days	—	1 42 3 36	6 05 10 55	10 10	No response to Reticulogen but optimum response to Campolon
9	1 36		4 84	1 18	108	0 32	8				Indirect +ve	4 5	50 5	0 0	Reticulogen 8 ml in 16 days	—	1 88 3 10	7 09 7 78	10 18	Optimum response.
10	2 84		10 03	1 21	110	0 32	3				-ve	3 6	13 0	0 0	Anahaemin 9 ml in 10 days	—	3 70	10 89	10 10	Optimum response
11	0 80		3 40	1 43	150	0 28	0				Indirect +ve	6 7	39 7	0 0	Examen 32 ml in 12 days	39/7	2 12 3 30	6 92 8 82	10 24	Sustained and optimum response but with larger doses
12	1 34		3 40	0 88	—	—	—				-ve	16 0	48 2	0 0	Examen 8 ml in 2 days	36/5	3 80	9 51	10 10	Optimum response to small dose
13	1 48		5 88	1 39	128	8 36	0				Indirect +ve	12 0	41 5	0 0	Anahaemin 20 ml in 10 days.	20/9	2 60 4 06	8 33 11 76	14 32	Optimum and sustained response
14	2 50		8 99	1 42	104	0 34	6				Indirect +ve	2 0	17 8	0 0	Examen 22 ml in 10 days	9/7	3 32 4 36	10 03 14 18	10 26	Optimum and sustained response
15	1 60		5 19	1 09	100	0 32	6				Indirect +ve	16 0	36 6	0 0	Examen 22 ml in 10 days	—	2 40 3 00	7 43 10 72	10 22	Optimum response

TABLE 1—Continued

Case No	R.B.C. in mill per cu mm	Hb in Gm per 100 ml	C I	M C V $\mu$	M C H C %	Vanden Bergh reaction	Sternal Puncture			Liver extract used and its total quantity	Max. reticulocytes % and the day	After treatment			Remarks
							Megaloblasts	Normoblasts	Pro-erythroblast			R B C in mill	Hb in Gm	Days	
16	2 48	10 38	1 42	113 0	36 9	—ve	7 0	31 4	0 0	Examen 10 ml in 4 days	—	2 2 1 8	10 38 8 65	10	No response W R + Lilly's Crude Liver 60 ml no response
17	1 92	7 43	1 36	105 0	38 0	—ve	1 5	9 5	0 0	Examen 26 ml in 12 days	—	2 96 4 00	9 51 12 97	14 30	Optimum response
18	1 20	5 53	1 54	137 0	33 5	indirect +ve	18	44 8	0	Examen 4 ml once	—	1 93	7 78	10	Suboptimum response
19	1 36	5 19	1 29	110 0	34 6	indirect +ve	—	—	—	Examen N.P. 10 ml in 10 days	—	2 64 3 25	6 92 12 11	10 25	Optimum response
20	0 68	2 42	1 23	132 0	24 8	indirect +ve	—	—	—	Examen N.P. 12 ml in 10 days	—	1 80 2 80	5 19 7 78	14 28	Optimum response
21	2 80	11 24	1 35	—	—	—	—	—	—	Examen N.P. 6 ml in 6 days	—	3 28	13 84	14	Optimum response
22	0 96	3 11	1 10	—	—	—	—	—	—	Examen N.P. 6 ml in 6 days	—	1 84 4 20	5 53 10 38	14 40	Optimum and sustained response
23	2 24	11 24	1 72	—	—	—	—	—	—	Examen N.P. 5 ml in 5 days Plexan 24 ml	—	2 34 2 40	10 38 10 52	14 28	No response but refractory to crude liver extract as well
24	1 60	6 92	1 53	—	—	—	—	—	—	Examen N.P. 6 ml in 6 days	—	2 93 2 92	10 89 10 72	10 17	Optimum but not sustained
25	0 75	4 32	1 93	—	—	—	—	—	—	Examen N.P. 6 ml in 12 days	—	1 80 2 60 3 20	7 09 9 51 11 07	10 16 38	Optimum response
26	1 38	6 05	1 51	—	—	—	—	—	—	Examen N.P. 6 ml in 6 days	—	2 68 2 64	10 38 10 38	10 17	Optimum response but not sustained
27	0 77	3 40	1 46	—	—	—	—	—	—	Examen N.P. 6 ml in 6 days + 6 ml after 14 days.	—	2 12 2 32 2 32	6 74 7 78 7 83	10 24 31	Optimum response but not sustained and did not respond further
28	1 04	5 19	1 75	—	—	—	—	—	—	Examen N.P. 6 ml in 6 days	—	3 20 3 16	10 03 11 24	14 21	Optimum and sustained
29	3 6	12 45	1 16	111 0	31 1	—	—	—	—	Examen N.P. 3 ml	—	4 32	12 80	14	Optimum
30	1 0	3 76	1 25	125 0	29 6	indirect +ve	—	—	—	Examen N.P. 3 ml	—	3 610	11 16	16	Optimum
31	1 0	2 76	0 90	105 0	26 2	indirect +ve	2 5	36	5 0	Examen N.P. 3 ml	—	0 9	3 11	16	No response. The patient left the hospital
32	1 82	7 95	1 47	—	—	—ve	4 0	34 5	12 0	Examen N.P. 1 ml	—	3 08	10 14	1	Optimum

TABLE 1—Concluded

Case No	R.B.C. in mill per cu mm	Hb in Gm per 100 ml	C I	M.C.V. cu $\mu$	M.C.H.C. %	Van den Bergh reaction	Sternal Puncture			Liver extract used and its total quantity	Max reticulocytes % and the day	After treatment			Remarks
							Megaloblasts	Normoblasts	Pro-erythroblasts			R.B.C. in mill	Hb in Gm	days	
33	1 09	4 84	1 49	163 4	25 8	indirect +ve	—	—	—	Examen N.P. 2 ml in 2 days	—	2 24 3 32	7 78 10 03	14 34	Optimum response to 2 ml
34	1 84	8 40	1 51	125 0	36 0	—ve	2 0	60 0	6 0	Examen N.P. 3 ml in 3 days	—	3 72	12 11	21	Optimum response to 3 ml
35	1 44	4 84	1 14	104 0	32 0	—ve	—	—	—	Examen N.P. 3 ml in 3 days	—	2 10	6 05	14	Suboptimum response
36	1 88	7 78	1 43	122 3	33 4	—ve	4 5	30 0	9 5	Examen N.P. 1 ml Proteolysed Exatropo 8 ml in 5 days	—	2 32 2 34 3 35	9 51 10 03 12 11	17 23 37	Suboptimum response No further response Responded to larger doses of crude liver extract.
37	0 96	4 32	1 51	156 2	28 6	—ve	6 5	27 5	1 5	Examen N.P. 2 ml	—	1 66 1 68	6 92 7 78	14 21	Suboptimum response No further response
38	1 28	5 19	1 30	142 5	28 8	—ve	17 6	34 6	1 2	Examen N.P. 2 ml	—	2 64 2 60	9 33 9 51	14 21	Optimum response No further response
39	1 84	8 60	1 59	127 0	34 4	—ve	16 8	8 8	0 0	Examen N.P. 2 ml	—	2 28	9 51	14	Suboptimum response
40	1 84	7 43	1 42	119 4	33 7	—ve	34 2	6 8	0 0	Examen N.P. 2 ml	—	2 28	10 03	14	Suboptimum response
41	1 40	7 78	1 89	157 1	35 3	indirect +ve	21 6	25 0	0 8	Examen N.P. 2 ml	—	2 24	10 38	14	Suboptimum response
42	1 16	5 36	1 56	136 1	33 9	indirect +ve	20 4	30 8	0 4	Examen N.P. 2 ml	—	2 40	7 78	14	Optimum response
43	0 92	4 84	1 77	139 1	37 8	indirect +ve	28 4	33 2	0 4	Examen N.P. 2 ml	—	2 24	9 18	14	Optimum response
44	1 48	6 92	1 69	135 1	36 1	indirect +ve	22 24	35 0	0 3	Examen N.P. 2 ml	—	1 64	6 92	14	No response Wassermann Reaction positive No response to crude liver extract.
45	2 04	10 38	1 75	151 9	33 4	—ve	12 0	28 84	0 5	Examen N.P. 2 ml	—	3 24	12 11	14	Optimum response

in the earlier cases by the reticulocyte response and improvement in the level of the red blood cells and of the hemoglobin during a period of ten days and in the latter part of the work by the formula of Dyke and Della Vida  $I = (0.93 - 0.214)EO$  where  $I$  is the average weekly increase in red cells during the first two weeks of treatment and  $EO$  is the red cell count before the treatment. Most of the investigations were carried out by us personally, a few gastric analyses were done by the clinical laboratory. The results are given in table 1 which should be consulted for the details of the case histories which follow.



in agreement with the findings of Trowell<sup>9</sup> and Sundaram<sup>10</sup> When given in larger total doses (larger than 12 ml), the response continues for a longer period

### *Group II—Twelve Cases Treated with Examen*

*Case 4* A 35 year old female complained of general weakness and stomatitis of six months duration RBC 1.88 mill per cu mm, Hb 8.99 Gm per 100 ml M.C.V. 127.0 cu  $\mu$  M.C.H.C. 39 per cent She was given 2 ml Examen The highest reticulocyte count was 4.2 per cent on the fifth day Improvement in ten days was optimum but was not maintained and the blood count fell slightly in the next ten days

*Case 18* A male aged 25 RBC 1.2 mill per cu mm., Hb 5.35 Gm per 100 ml M.C.V. 137 cu  $\mu$ , M.C.H.C. 33.5 per cent Response to 4 ml of Examen in ten days was optimum

*Case 12.* A male aged 24 years was suffering from diarrhea stomatitis and attack of vomiting of one month's duration Examination of the blood showed RBC 1.34 mill per cu mm Hb 3.40 Gm per 100 ml He was treated with 8 ml of Examen given in the first two days A maximum reticulocyte response of 36 per cent was recorded on the fifth day and the improvement was optimum

*Case 16* A 30 year old male was admitted for intermittent diarrhea for the previous three months and low fever and general weakness of six weeks duration RBC 2.48 mill per cu mm Hb 10.38 Gm per 100 ml M.C.V. 113.0 cu  $\mu$  M.C.H.C. 36.9 per cent He had a complicating ulcerative colitis and his blood Wassermann reaction was positive There was a histamine-fast achlorhydria He did not respond to Examen 10 ml given in the first four days neither did he respond afterwards to 60 ml (5 ml daily) of crude liver extract (Lilly) He was given arsenic injections for his syphilis while he was having crude liver extract He left the hospital against advice without any improvement

### *Comment on the Cases in Group II*

Cases 3, 5, and 6 responded well to 12 ml to 14 ml of Examen Case 7 responded poorly to 20 ml of Examen but subsequently showed a better, though not an optimum response, to Campolon 75 ml given in twenty days When Examen was given in large doses of 22 ml in Case 14 and Case 15, of 20 ml in Case 17, and of 32 ml in Case 11, the response was not only adequate but persistent for many days afterwards In this series of 12 cases of T.M.A. treated with Examen, 2 showed adequate response to a small dose of 2 ml and 4 ml, and, 3 to a total dose of 12 to 14 ml, but neither these dosages were able to sustain the improvement beyond a period of ten to fourteen days In 4 patients, a larger dosage of 22 ml to 32 ml was not only able to produce a good response, but this response was maintained for days afterwards In 1 case, 10 ml of Examen did not produce any improvement, but later this case did not respond to crude liver extract either Another showed a poor response to 20 ml of Examen, he was immediately afterwards treated with Campolon (Bayer), 75 ml But even with Campolon the response was comparatively poor and slow

### *Group III—Two cases of T.M.A. Treated with Reticulogen*

*Case 8* A male aged 17 years complained of recurring attacks of stomatitis and low fever for four months He suffered from diarrhea while under observation in the wards The spleen was palpable and there was a hemic murmur in the precordial area RBC 1.46 mill per cu mm Hb 6.05 Gm per 100 ml M.C.V. 115.0 cu  $\mu$  M.C.H.C. 34.4 per cent The Van den Bergh reaction was indirect positive He was treated with Reticulogen 0.5 ml daily for eight days The response was poor Subsequently he responded well to Campolon

*Case 9* A male aged 38 years complained of general weakness of two months duration There was history of diarrhea the previous year He was very poorly nourished The spleen and the liver were both palpable RBC 1.36 mill per cu mm Hb 4.84 Gm per 100 ml M.C.V. 108 cu  $\mu$  M.C.H.C. 33.8

per cent The Van den Bergh reaction was indirect positive He was treated with Reticulogen 8 ml in eight days the response was adequate

#### *Group IV—Cases of T M A Treated with Examen (New Potency)*

Towards the end of 1944 Emery and Hurran<sup>2</sup> had prepared a refined liver extract which contained a minimal amount of solids and was consistently effective in a small dosage in Addisonian pernicious anemia One ml was extracted from 60–80 Gm of liver This liver extract (Examen New Potency [N P]) has been used by us in the treatment of 27 cases of T M A since 1945 It was used in varying doses 10 ml in Case 19 12 ml in Case 20 and 8 ml in Case 25 it produced an optimum response in all In 2 out of the 3 the response was maintained for a few days afterwards Six ml of Examen (N P) (1 ml daily for first six days) was given to 6 cases (Nos 22, 24 25 26 27 and 28) In 2 (Nos 22 and 25) not only did it produce an optimum response but the effect was maintained for a further period of about three weeks In the remaining 4 cases (Nos 24 26 27 and 28) the same dosage produced an optimum response In Case 23, 5 ml of Examen (N P) did not produce any response Later Plexan crude liver extract 24 ml was equally ineffective In 5 cases (Nos 29 30 31 34 and 35) Examen (N P) was administered in the dosage of 1 ml for the first three days a total of 3 ml for each case In 3 (Nos 29 30 and 34) there was an optimum response in 1 (No 35) a suboptimal and in 1 (No 31) no response at all Case 31 was admitted in the general hospital 15 days after delivery (a stillborn child) displaying pregnancy anemia She left the hospital without further treatment Ten cases (Nos 33 37 38, 39 40 41 42 43 44 and 45) were treated with 2 ml of Examen (N P) It produced an optimum response in 5 cases (Nos 33 38 42 43 and 45) suboptimum response in 4 (Nos 37 39 40 and 41) and no response in the last case (No 44) Case 33 not only showed an optimum response to 2 ml of Examen (N P) but the response continued for a period of thirty four days Case 44 who had a positive Wassermann reaction failed to respond to 12 ml of potent crude liver extract subsequently In Cases 32 and 36 1 ml of Examen (N P) produced an optimum and suboptimal response respectively (Cases 30 32, 33 and 34 have been the subject of a separate report by one of us<sup>12</sup>)

#### *Comment on the Cases in Group IV*

From the above findings we conclude that Examen (N P) produced an adequate hemopoietic response in all cases with the exception of 3 (Cases 23, 31 and 44) A total dose of 2 or 3 ml seems adequate to obtain an optimum response in the majority of cases In an occasional case, even 1 ml has given satisfactory results When given in the larger doses of 6 ml, not only is the response satisfactory but its hemopoietic effect continues for many days afterwards

#### DISCUSSION

No better initial response is likely to be obtained in an uncomplicated case of Addisonian pernicious anemia by giving a dose larger than is necessary to produce an optimum response as defined above (Emery and Hurran<sup>2</sup>) Probably the same statement is applicable to the treatment of T M A In a total of 45 cases of T M A treated with refined liver extract, 39 responded satisfactorily, 32 gave an optimum response and 7 suboptimum response The 6 cases which did not respond to refined liver extract were as follows Case 8 did not respond to Reticulogen but later responded satisfactorily to crude liver extract Cases 7, 16, 23 and 47 not only did not respond to refined liver extract but failed as well to respond to crude liver extract administered subsequently Case 31, pregnancy anemia, did not respond to refined liver extract during her stay of one week but then she did not stay in the hospital long enough for detailed observations There were only 2 cases, then, which did not really respond to refined liver extract

The above observations are in general agreement with those of Foy and Kondi,<sup>7</sup> Fairley,<sup>8</sup> Trowell<sup>9</sup> and Sundaram,<sup>10</sup> but differ from their findings in that the dose found effective in our series is much smaller than that used by any of them.

Some textbooks<sup>15 16</sup> recommend that any liver extract, no matter of what type, should be given in a certain dose volume (e.g., 2 ml or 4 ml twice a week) in order to obtain a satisfactory remission in Addisonian pernicious anemia and the allied conditions. Dyke and Della Vida<sup>12</sup> have adopted the arbitrary criterion that any liver extract producing a good response with a total dosage of less than 10 ml over the first fortnight should be regarded as sufficiently potent for therapeutic use. There are no similar criteria available for T M A. If the above criteria are applied to T M A it can be deduced, from our results, that refined liver extracts used in this series were definitely of therapeutic value, particularly the Examen (N P). But, as Emery and Hurran<sup>3</sup> have pointed out, the volume is not by itself an indication of its potency. It is the therapeutically active principle of the solid content which is the deciding factor, and this can be dissolved in varying quantity of the fluid-base. In the absence of a reliable and generally accepted *unitage* in assaying the potency of liver extracts,\* the important point is the quantity of the original liver from which the active solids have been extracted. The difference between the efficacy of crude and refined liver extract, expressed in terms of *original liver*, should be the ultimate basis for discussion. Originally it was found that in the treatment of Addisonian pernicious anemia, crude extract derived from 60 to 80 Gm of liver produced a satisfactory response, whereas the equivalent of 200 Gm of the original liver was required when administered in the form of a refined liver extract. In the process of purification, activity might be lost in the discarded side fractions, it might even be totally destroyed. Or, the hemopoietic activity of a liver extract may be dependent on the simultaneous presence of more than one substance as postulated by Jacobson and Subbarow<sup>17</sup> some years ago. Possibly some of these factors might be removed during the concentration or purification. Emery and Hurran<sup>3</sup> claim to have produced a refined liver extract (Examen N P) containing in 1 milliliter, the equivalent of 60 to 80 Gm of liver, which in 1 ml dosage was effective in 3 cases of Addisonian pernicious anemia, thus removing the discrepancy between the response to refined and crude extracts in terms of the original liver.

The average amount of crude liver extract required to produce a satisfactory response in T M A varies with the brand. Twenty ml (equivalent to 100 Gm of original liver) of Campolon was used by Napier<sup>1</sup> and by Wills and Evans.<sup>4</sup> Twelve ml of Plexan (equivalent to 90 Gm of original liver) and 8 ml of Chemilon (Dr Rao's laboratory) (equivalent to 100 Gm of original liver) were considered necessary to produce a satisfactory response by Patel.<sup>12</sup> This shows that the crude liver extract derived from about 90 to 100 Gm of original liver is effective in an average case of T M A. One to 2 ml of Examen (N P) which is found to be effective in T M A will be derived from 60 to 120 Gm of the original liver. Thus, the discrepancy between the response to refined and crude liver extracts in

\* The United States Pharmacopoeia has developed standards for *unitage* of liver extracts which appear to be reliable and are based on assays in human cases of Addisonian pernicious anemia. *Edit*

T M A in terms of the original liver, which was so noticeable in the past, is now insignificant. It will be noticed that the amount of active principle (original liver) needed to produce a satisfactory response is more than that required in Addisonian pernicious anemia, but otherwise the deficiency in the majority of cases of T M A seems to be similar to that in Addisonian pernicious anemia. Moore et al.<sup>2</sup> working in the U S A discussing their successful treatment of N M A with highly 'purified' liver extract (Reticulogen) concluded that either (1) the N M A found in the United States differed in some fundamental respect from the N M A seen in India, or, that (2) the 'purified' liver extract used by them contained some hypothetic substance not found by Wills<sup>4</sup> in Anahaemin. From the therapeutic results obtained in the present series it can be reasonably stated that tropical macrocytic anemia in India does not differ fundamentally from the nutritional macrocytic anemia found in the United States or elsewhere.

Obviously, then, calculating in terms of original liver and comparing the responses obtained by the "crude" and the newer "refined" extracts in Addisonian pernicious anemia and in T M A it can be inferred that a considerable amount of active principle was probably not extracted in the older "refined" liver extracts, and, hence, the discrepancy in the results obtained in the past both in Addisonian pernicious anemia and T M A. This might, possibly also, be the explanation of the conflicting results obtained in the past by various workers in the treatment of T M A with refined liver extracts. Fairley<sup>8</sup> believed that there was no advantage—on the contrary a disadvantage of a higher cost—with the use of refined liver extract in the treatment of T M A. This is true if one has to give large doses, but if 3 ml or less be considered a satisfactory dose to obtain an optimum response, the cost will not be much higher and there would be the added advantage of less pain locally and of reduction in the systemic reactions to the injections of the liver extracts.

#### SUMMARY

- 1 A series of 45 cases of T M A treated with refined liver extract is reported.
- 2 Refined liver extract was found to be effective in 39 cases.
- 3 It was found that 2 or 3 ml of refined liver extract (Examen N P) was sufficient to produce an optimum response.
- 4 As judged from therapeutic observations, it is suggested that in the majority of cases of T M A the deficiency is similar to that in Addisonian pernicious anemia, though the mode of production of the deficiency may not be the same.

#### ACKNOWLEDGMENT

Our thanks are due to Dr R. Row, Hon. Director P G. Singhance Hindu Hospital Bombay where all the cases were treated for permission to publish these reports, and the firms concerned for the generous gift of liver extracts.

#### REFERENCES

- <sup>1</sup> NAPIER L E The aetiology of tropical macrocytic anaemia. *Ind Med Gaz* 74: 1-10 1939
- <sup>2</sup> MOORE C V, VILTER R, MINNICH V AND SPIES T D Nutritional macrocytic anaemia in patients with pellagra or deficiency of the vitamin B complex. *J Lab & Clin Med* 25: 1-6-1-55 1944



- <sup>3</sup> EMERY W R AND HURRAN, W J The nature of injectible liver extracts Brit M J 1 75-78 1945
- <sup>4</sup> WILLS L CLUTTERBUCK P W, AND EVANS, B D F A new factor in the production and cure of certain macrocytic anaemias Lancet 1 311-314, 1937
- <sup>5</sup> WILLS L AND EVANS B D F Tropical macrocytic anaemia Its relation to pernicious anaemia Lancet 11 416-424 1938
- <sup>6</sup> NAPIER L E DAS GUPTA, C R, CHAUDHARI R N SEN G N, CHAUDHARI RAI, M N SEN GUPTA P C AND MUZUMDAR D N Anahaemin in tropical macrocytic anaemia Ind Med Gaz. 73 385-390 1938
- <sup>7</sup> FOY H AND KONDI, A Response of nutritional macrocytic anaemia to anahaemin Lancet 11 360-363 1939
- <sup>8</sup> FAIRLEY, N H Tropical macrocytic anaemia in an Indian treated with anahaemin Lancet 1 1118-1119 1940.
- <sup>9</sup> TROWELL, H C Deficiency anaemias of malnutrition Lancet 1 43-46 1943
- <sup>10</sup> SUNDARAM S K. Refined liver extract in the treatment of nutritional macrocytic anaemias Ind Med Gaz 79 253-256, 1944
- <sup>11</sup> BHENDE Y M AND PATEL, J C Studies in anaemias Indian Physicians 44-45 1946
- <sup>12</sup> PATEL J C Crude and refined liver extract in nutritional macrocytic anaemia Ind Med Gaz 81 452-454 1946
- <sup>13</sup> DYKE S C AND DELLA VIDA B L Maximal response to liver therapy in pernicious anaemia Lancet 11 275-278 1942.
- <sup>14</sup> BHENDE, Y M Achlorhydria and anaemia Ind Med Gaz 77 13-15, 1942.
- <sup>15</sup> WITTS, L J Text book of Practice of Medicine edited by Price F W, ed 5 London, Oxford University Press 1941 P 821
- <sup>16</sup> BEAUMONT, G E AND DODDS, E C Recent Advances in Medicine, ed 11 London, Churchill, 1943 P 311
- <sup>17</sup> JACOBSON, B H AND SUNBAROW Y Studies of the principle in liver effective in pernicious anaemia J A M A 116 367-374 1941

## A CASE OF CYCLICAL AGRANULOCYTOSIS WITH MARKED IMPROVEMENT FOLLOWING SPLENECTOMY

By H W FULLERTON, M D , M R C P , AND H L D DUGUID, M B , CH B

IN 1946, Vahlquist<sup>1</sup> described a case of true cyclical agranulocytosis and summarized the findings in the other 5 cases reported in the literature before that time. Since then, the number has been increased to 7 by an example published in 1946 by Reznikoff.<sup>2</sup> In 5 of these cases, the condition had commenced in infancy and was characterized by periods of complete or almost complete disappearance of neutrophil leukocytes from the peripheral blood at regularly recurring intervals of approximately 21 days. Pyrexia and ulceration of the mouth were the main features of the attacks. Of the other 2 published cases, one was a girl of 18 years<sup>3</sup> and the other a woman of 43 years.<sup>4</sup> Some examples of agranulocytosis occurring repeatedly at the time of menstruation have been properly excluded from the group of idiopathic cyclical agranulocytosis because the possibility of the women affected having taken drugs (e.g., amidopyrine) at the onset of menstruation was not excluded.

From a perusal of the published case reports it is obvious that the characteristic features of cyclical agranulocytosis have been intriguing enough to stimulate much investigation into the pathogenesis. The report by Imerslund<sup>5</sup> is the most striking example of the extensive nature of the investigations which have been performed in the study of this peculiar condition. Her patient, a boy of 16 years who had suffered from the disease since the age of 14 months, was subjected to very full hematologic, biochemical, bacteriologic and endocrinologic studies, but no abnormalities which could clearly be correlated with the leukocytic changes were discovered. Therapeutic efforts have been equally energetic and varied. Pent-nucleotide, blood transfusion, yellow bone marrow, the various vitamins, liver extract, anterior pituitary extract, ultra-violet light and short-wave therapy are some but not all of the measures which have been used, and none of them has succeeded in preventing or even in significantly modifying the persistently regular occurrence of agranulocytosis and the associated symptoms. Splenectomy has been done in only one of the reported cases, and was followed by no notable improvement.

The patient to be described is apparently unique, first because he is a man who developed cyclical agranulocytosis in advanced adult life, and second, because splenectomy has greatly modified the recurrent falls in the neutrophil leukocytes and has abolished his symptoms completely.

### CASE REPORT

J S, male, age 62 years was admitted to Aberdeen Royal Infirmary on June 6 1946

From the Department of Medicine, University of Aberdeen Aberdeen Scotland

**History of present illness** In October 1945 he thought he had fever felt generally unwell and had a course of sulphadiazine tablets (total 28 Gm) In January 1946, he had a painful throat and conjunctivitis and underwent another course of sulphonamides Sore throat recurred 10 February 1946 and, in addition, he developed boils to the neck and an ischio-rectal abscess Another course of sulphadiazine was taken at this time In March he again had pain in the throat and for the fourth time had sulphadiazine orally

Unfortunately, it was impossible to obtain exact details of these illnesses but the patient was quite definite that on each occasion infection had developed *before* sulphonamide was taken

Shortly before admission to hospital the ischio-rectal infection recurred and he had sore throat

**Past history** Seventeen years ago he developed rheumatoid arthritis which was treated by tablets, he had no injections This kept him from his work as a laborer for five years He was then able to work and had little disability except for occasional stiffness and pains in the knees for which he has taken aspirin During the 1939-45 war he was a cement worker

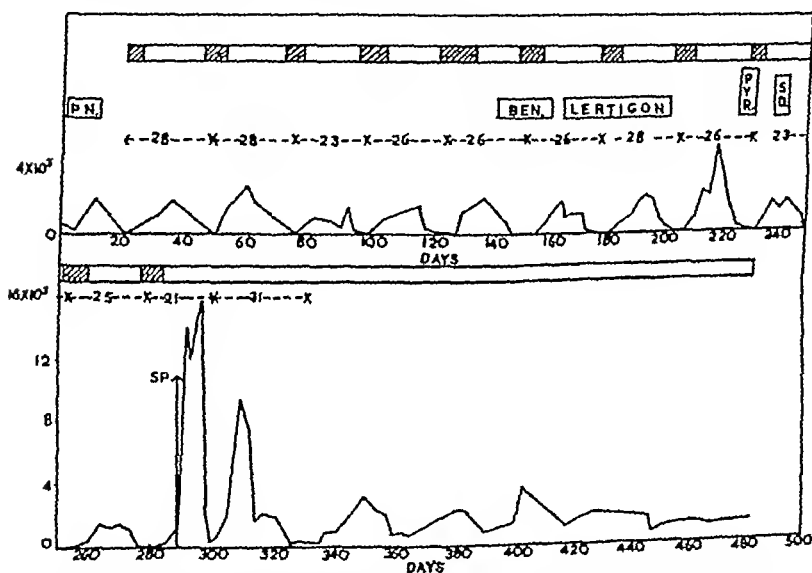


FIG. 1. CHART SHOWING VARIATIONS IN THE ABSOLUTE NUMBER OF NEUTROPHIL POLYMORPHS  
PN represents penicillin-nucleotide therapy, BEN benadryl, PYR. pyridoxine, SD sulphadiazine, SP splenectomy. The cross-hatched areas represent periods of pyrexia.

**Family history** Two brothers, one is alive and well the other died of consumption at the age of 40. His only sister is dead cause of death unknown to patient. Mother died at 38 years cause unknown. Father died in his early forties as a result of pneumonia. The patient is married and his two sons and one daughter are alive and well.

**Physical examination** He was a well-nourished well-colored man. There was some thickening about both wrists and the metacarpo-phalangeal joints with slight limitation of movement. B.P. 155/85. The heart was not enlarged and the sounds were normal. Examination of the various systems revealed no notable abnormalities, in particular it was noted that the spleen was not palpable.

**Course of the illness** Between June 1946 and March 1947, the patient suffered from twelve attacks in each of which the neutrophil polymorphs disappeared entirely from the peripheral blood usually for a period of four to five days. The intervals between the attacks were remarkably regular, varying from twenty-three to twenty-eight days (see fig. 1). Each attack was characterized clinically by malaise, anorexia, drowsiness, headache, pyrexia and variously localized infections. Inflammation and edema of the throat and

tonsils were constant features of the attacks. Conjunctivitis, iritis, blepharitis, recurrences of the ischio-rectal infection, painful superficial ulcers on the gums and tongue and areas of acute inflammation to the skin occurred in various combinations. Meatal depression became more marked with each succeeding attack. As the granulocytes reappeared in the blood, the various infections rapidly disappeared and the pyrexia settled, the patient's well being was quickly restored and he helped cheerfully in carrying out light work in the ward.

The variations in the absolute number of neutrophil polymorphs are presented graphically in figure 1, which shows also the time intervals between the midpoints of the phases of agranulocytosis. It is to be

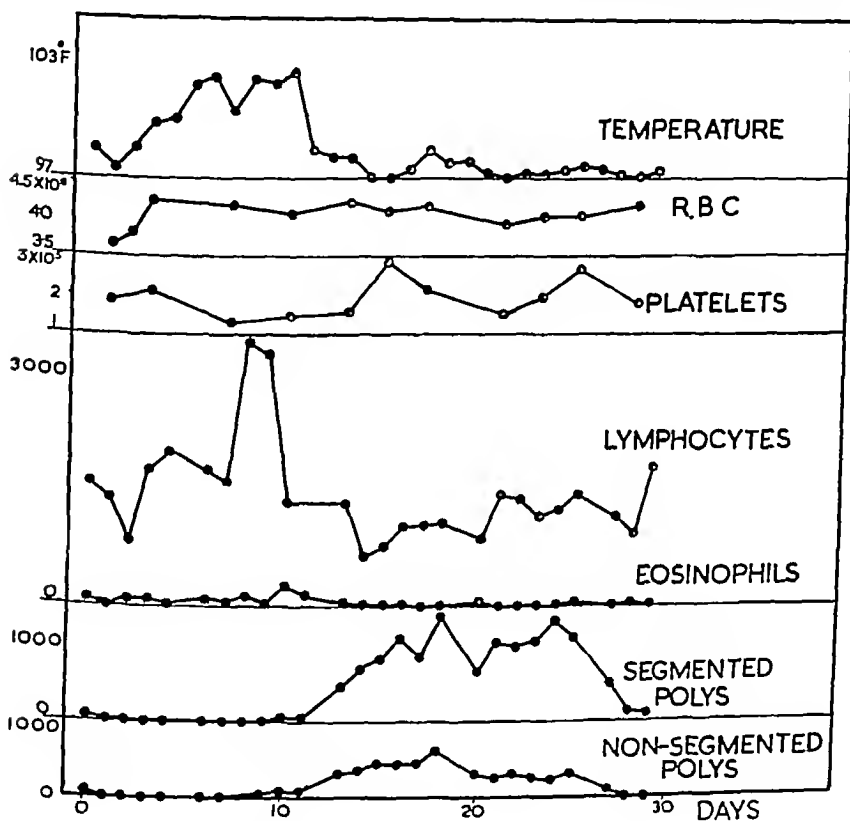


FIG. 2. CHANGES IN ALL COUNTS DURING ONE TYPICAL CYCLE

noted that between attacks the neutrophil polymorphs did not reach normal but were between 1 and 3 thousand per cu. mm. except on one occasion (217th day) when a figure of nearly 6000 was reached; this coincided with a recurrence of the ischio-rectal infection and was the only occasion on which infection occurred apart from the phases of agranulocytosis.

**Laboratory investigations.** The blood Wassermann reaction was negative. Throat swabs usually gave a growth of staphylococcus aureus. Blood cultures were sterile. X-ray of chest was normal. X-ray of the nasal sinuses showed chronic infection of the antra. Blood counts were done almost daily throughout the patient's stay in hospital.

In the period before splenectomy, in March 1947 (290th day) the outstanding feature was the comp-

disappearance of neutrophil polymorphs for four to five days every three to four weeks. The variations in these and other cells throughout one complete cycle are presented in figure 2. It is to be noted that the disappearance of the polymorphs preceded the rise of temperature. Throughout the presplenectomy period the hemoglobin and red cells showed no significant variations from a level of 80 per cent (Haldane) and 40 millions. The platelets varied between 100 000 and 350 000 per cu mm and although the lowest figures were found during phases of agranulocytosis considerable variation occurred between attacks. The eosinophil polymorphs varied from 0 to 700 per cu mm but their number showed no correlation with the neutrophil polymorphs. The lymphocytes always increased during an agranulocytic phase the extent of the increase varied with a maximum of 6 700 per cu mm. The basophils and monocytes showed no notable changes.

## SPECIAL INVESTIGATIONS

*Bone marrow studies* Because it seemed important to determine whether the periodic disappearance of neutrophil polymorphs was due to a failure in the produc-

TABLE 1—Sternal Marrow Differential Counts

	29/10/46	2/11/46	6/11/46	9/11/46	12/11/46	17/11/46	23/11/46	27/11/46
Myeloblast	0.8	0.8	2.4	4.4	4.0	2.8	0.8	1.2
Premyelocyte	1.6	1.2	6.0	7.6	7.2	3.6	0.8	1.2
Myelocyte { N	1.2	0.4	1.6	4.0	8.4	14.8	0	0
{ E	3.2	1.6	2.8	1.2	0	2.4	1.2	3.2
{ B	0	0	0	0.4	1.6	0.4	0	0
Metamyelocyte { N	0.8	0.4	0.1	6.0	25.4	36.8	11.6	0
{ E	7.2	7.6	5.2	2.4	2.0	2.0	2.8	2.0
{ B	0.4	0	0	0	0.4	0	0	0
Segment { N	3.2	2.8	0	2.8	4.0	7.6	13.2	0.8
{ E	6.4	4.0	4.0	7.2	3.2	0.4	0.8	3.2
{ B	0.8	0	1.6	2.0	1.6	0.4	0	0.8
Lymphocyte	37.0	33.6	43.9	39.6	21.4	15.2	26.0	34.4
Plasma cell	0.8	3.6	6.0	0.4	4.5	4.4	4.0	3.6
Monocyte	0.8	2.4	1.2	5.6	3.2	0	3.2	4.8
Hemocythlast	0.4	0	0.8	0	0	0	0.8	1.2
Normoblast	35.4	41.6	23.6	16.0	13.2	9.2	34.8	43.6
Megakaryocyte	0	0	0.8	0.4	0	0	0	0
Absolute number of neutrophil polymorphs in blood	110	0	0	235	1010	1440	1370	90

tion of these cells by the marrow, or to their excessive destruction after delivery into the blood, eight aspirations of sternal marrow were performed at intervals of a few days throughout one typical cycle. The differential counts of the nucleated marrow cells are presented in table 1. It is to be noted that the percentage of normoblasts varies between 9.2 and 43.6. Since no significant variations occurred in the red cell count, it may be presumed that the absolute number of normoblasts remained fairly constant and the variation in the percentage figures is due simply to changes in the other marrow cells, especially the neutrophil polymorphs and their precursors. In other words, the total cellularity of the marrow is greatest when the percentage of normoblasts is lowest, and the absolute increase in the granular cells is of greater degree than is indicated by the percentage figures in the table. In figure 3, the neutrophil cells and their precursors in the mar-

row have been charted so that their course can be compared with simultaneous variations in the number of neutrophil polymorphs in the blood. It is clear that a rise, first in myeloblasts and premyelocytes and then in myelocytes, in the marrow precedes the appearance of neutrophil polymorphs in the blood, and the early forms of the myeloid series decrease in the marrow a few days before the fall of neutrophil polymorphs in the blood. It follows that the underlying cause of the phases of agranulocytosis was a periodic failure of the marrow to produce neutrophil poly-

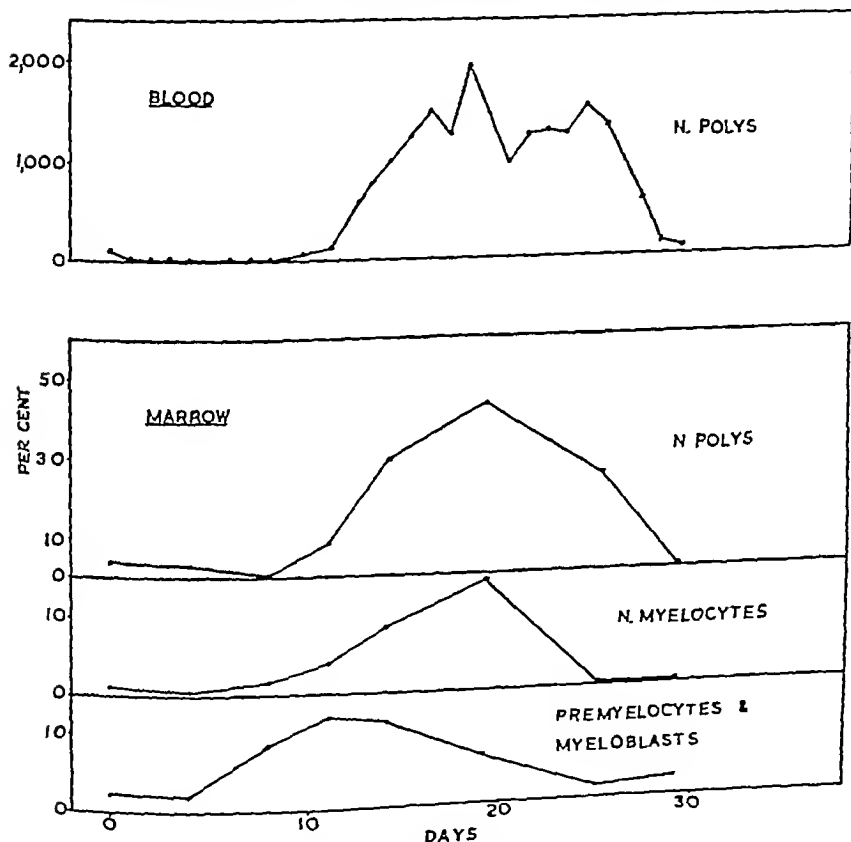


FIG. 3. CHART SHOWING CORRELATION BETWEEN MARROW FINDINGS AND BLOOD LEUKOCYTE COUNT DURING A TYPICAL CYCLE

morphs, and it is unnecessary to postulate any increase in their destruction to explain the findings. There is no evidence of a maturation defect in the marrow since even the myeloblasts are markedly decreased in the agranulocytic phase. It may be pointed out, however, that if reliance had been placed on a single marrow examination wrong conclusions might have been drawn. For example, on the eleventh day of the cycle studied (see fig. 3), a considerable number of myeloblasts and premyelocytes (12.0 per cent of the marrow cells) were present with few

myelocytes and polymorphs and only 135 neutrophil polymorphs per cu mm in the blood. This could have been interpreted as a maturation defect in granular cell formation, whereas the true explanation is that formation of mature cells from the regenerating myeloblasts and premyelocytes had not yet occurred at the time of the aspiration. We believe that erroneous conclusions have been drawn by several writers from the results of a single marrow examination in agranulocytosis.

*Adrenaline Test* During an agranulocytic phase and mid-way between two attacks, 10 cc adrenaline was injected subcutaneously and white cell counts were performed at intervals after the injections. In the phase of agranulocytosis no significant change in the neutrophil polymorphs was found following the injection (before injection, 23 neutrophil polymorphs per cu mm, maximum after injection 106), whereas between attacks the neutrophil polymorphs were increased from 1670 to a maximum of 2600, 55 minutes after the injection.

*Transfusion of Patient's Plasma* In an attempt to discover if a factor with a depressant action on leukopoiesis circulated in the patient's plasma at regular intervals coinciding with the phases of agranulocytosis, one pint of blood was removed from the patient on two occasions (at the beginning of an attack and mid-way between attacks) and the plasmas were injected intravenously into another subject. On both occasions no significant effect on the recipient's leukocytes occurred. It is realized that this experiment is a crude one and that the negative result does not rule out the possibility that a depressant factor might have been demonstrated by suitable animal experiments.

*Sex Hormone Excretion* The regular occurrence of the crises in cyclical agranulocytosis has naturally led to a comparison with menstruation, and to the suggestion that the underlying disturbance might be a rhythmical disturbance of sex hormone production. Imerslund<sup>6</sup> had prolactin and folliculin titrations in the urine done every other day throughout one cycle in her patient, a boy of 16 years. Three times, the excretion of folliculin was a little greater than the normal range, on the first and third of these occasions the polymorphs were low, on the second they were high. No notably abnormal values for prolactin excretion were found. Thompson<sup>7</sup> considered that in his patient, a man of 25, the number of the neutrophil polymorphs could be correlated with the urinary excretion of female sex hormone but as the investigations were continued during only a short part of one cycle and were not repeated, the findings are not convincing. Perhaps the strongest evidence against the sex hormone theory is provided by the cases of Embleton<sup>4</sup> and Doan,<sup>3</sup> the only true examples of idiopathic cyclical agranulocytosis we have been able to discover in women of reproductive age. In both cases, no relationship could be observed between the times of menstruation and the occurrence of phases of agranulocytosis. We were unable to have estimations of prolactin and folliculin excretion carried out in our patient but the urinary 17-ketosteroids were estimated twice, at the beginning of an attack a figure of 7.3 mg/24 hours was obtained and mid-way between attacks the figure was 7.5 mg.

#### TREATMENT

*Penicillin* As each attack of agranulocytosis developed, our patient was given penicillin intramuscularly and this was continued until the neutrophil polymorphs

had reappeared in significant numbers, usually a total of approximately 2 million units was given over a period of about ten days.

*Pentnucleotide* Intramuscularly was given in the first attack observed in hospital (see fig. 1). No clear-cut effect was produced and as undesirable reactions followed the injections, this treatment was not repeated.

*Anti-histamine drugs* In intermittent hydrarthrosis effusion into the affected joint often occurs with remarkable regularity, so that in this respect at least the condition is similar to cyclical agranulocytosis. The possibility that an allergic disturbance may be responsible for intermittent hydrarthrosis and the fact that this mechanism has been held to explain some cases of agranulocytosis<sup>7, 8</sup> led us to try the effects of benadryl and lertigon in our patient. Benadryl was given in a dose of 250 mg daily from days 140 to 152 (see fig. 1). The drug was purposely started at a time when the neutrophil polymorphs had started to fall. No influence on the usual course of the illness was observed. Lertigon (histamine azoprotein, Parke Davis and Co.) was commenced on day 161 in a dose of 0.01 cc, which was gradually increased to a maximum of 1.25 cc on day 200. Again the pattern of the polymorph curve was undisturbed.

*Pyridoxine* In view of recent reports of the efficacy of pyridoxine in the treatment of acute agranulocytosis,<sup>9-11</sup> this drug was given intramuscularly (total 300 mg) and orally (total 450 mg) over a period of six days. Treatment was started on day 224 while the neutrophil polymorphs were falling (see fig. 1). The dosage employed was rather less than that usually recommended but the complete absence of any influence on the course of the polymorphs makes it unlikely that larger doses would have been effective.

*Sulphadiazine* Prior to admission to hospital our patient had taken several courses of sulphadiazine. On each occasion, infections had developed before sulphadiazine was taken and the attacks of agranulocytosis continued for many months after the last doses of the drug, so that it seemed most unlikely that sulphadiazine had played any part in causation. However, to confirm this view, a total of 25 Gm sulphadiazine was given orally (days 237-241). Again the usual course of the polymorphs was unchanged.

*Splenectomy* The decision to perform splenectomy cannot be regarded as having a very rational foundation, it was based on the consideration that the role of the spleen in regulating the numbers of the various formed elements in the blood is not yet fully understood, and that long continued leukocytosis may follow splenectomy. We were familiar with the reports of cases of chronic (noncyclical) granulocytopenia in which the operation has proved successful.<sup>1-16</sup> In such cases, however, the general view is that the granulocytopenia is due to excessive phagocytosis of polymorphs in the spleen, and Wiseman and Doan<sup>17</sup> include splenomegaly and hyperplasia of the myeloid series in the marrow among the diagnostic criteria. In our patient there were several important differences: the spleen had never been palpated, in the phases of agranulocytosis there was aplasia of the myeloid series in the marrow, and the results of serial marrow and blood studies provided no evidence that excessive peripheral destruction of neutrophils occurred. At the time when our decision was made we had found no record of splenectomy in true cyclical agranulocytosis, the report of Reznikoff<sup>2</sup> was not at that time available to us.



His patient was a boy of 18 years who, since infancy, had suffered from cyclical agranulocytosis. Following splenectomy the absolute numbers of neutrophil polymorphs in the blood were not significantly altered, although the degree of prostration during the leukopenic phases was considered to be less.

Splenectomy was performed on the 290th day by Mr G Gordon Bruce. No accessory spleens were found. Dr W M Davidson reported on the spleen as follows: The spleen was enlarged to some three or four times the normal size and was of a firm consistency. The cut surface was fairly uniform, the malpighian corpuscles and trabeculae being visible but not prominent. Microscopically, there were no outstanding features. A small amount of iron pigment was present but there was neither erythrocyto- nor leukophagocytosis to be seen. The central arteries of the malpighian corpuscles showed a hyaline degeneration, and this had extended into the small vessels in the germ center. A slight degree of fibrosis was present in the pulp tissue and, at one or two points, small collections of cells suggested foci of hemopoiesis, but not very definitely. There was no amyloid change.

The operation was followed quickly by a marked leukocytosis which reached a maximum of 18,900 with 83 per cent neutrophil polymorphs five days after operation (see fig 1). Thereafter, the white cells fell rapidly but, in contrast with every phase of leukopenia observed before operation, the neutrophil polymorphs did not completely disappear, after falling to 406 per cu mm (day 299) they commenced to rise again. Another marked fall occurred about a month later (lowest number of neutrophil polymorphs 90 per cu mm on day 326), but since then, although fluctuations have occurred, the lowest number of neutrophil polymorphs has been 640 per cu mm on day 447. Thus, splenectomy has not been followed by a normal white cell picture. Fluctuations of considerable magnitude have occurred but at no time have the neutrophil polymorphs completely disappeared from the peripheral blood, and this change has been accompanied by a great improvement in the patient's health, since no episodes of pyrexia and no infections have developed in the period of 190 days during which he was carefully studied following the operation. Two months later (254 days after operation) the patient was seen again and reported continued good health and freedom from infections. The white cell count was 4050 per cu mm with 16 per cent neutrophil polymorphs (648 per cu mm), Hb 106 per cent, R B C 520, C I 1 or 1\*.

#### DISCUSSION

Investigations in the case described above failed to reveal a clear cause for the spectacular variations in the neutrophil polymorphs. It cannot be doubted that splenectomy modified the course of the illness markedly, although neutropenia of considerable degree has persisted. This suggests the possibility that the spleen was only one site of a more widespread lesion† but its nature and the mechanism whereby it produced cyclical variations in the myeloid series of cells remain entirely obscure.

\* *Addendum* When last seen 2/2/49 the patient had remained free from infections and the leukocyte count was 8500 per cu mm neutrophil polymorphs 47 per cent, eosinophils 6 per cent, lymphocytes 31 per cent, monocytes 16 per cent.

† Or that its removal resulted in removal of the normal inhibitory effect of the spleen on white cell delivery from the bone marrow to the blood. *Ed*

Judging by the small number of cases reported in the literature, cyclical agranulocytosis appears to be a very rare disease. We feel, however, that it may be more common than is realized because cases may easily be missed. It was only after our patient had been in hospital for several months and the absolute numbers of neutrophil polymorphs had been charted, that the striking cyclical nature of the disturbance was appreciated. Frequent white cell counts over a considerable period are essential if the condition is to be recognized. In several of the cases reported in the literature as examples of chronic granulocytopenia, white cell counts were done so infrequently that cyclical variations, if they were present, could not have been detected. More careful study of patients with chronic leukopenia, particularly if there is a history of recurrent episodes of ulceration of the mouth and other infections, would probably reveal more examples of true cyclical agranulocytosis.

### SUMMARY

1. A case of cyclical agranulocytosis beginning in a man at the age of 62 years is described.
2. The course of the illness was greatly modified by splenectomy, neutropenia continued but phases of complete agranulocytosis and infection ceased.

### REFERENCES

- 1 VAHLQUIST, B. Cyclical agranulocytosis, Report of a case with a short survey of the disease. *Act. Med. Scandinav. Suppl.* 170: 531-542, 1946.
- 2 REZNIKOFF, P. Cyclical neutropenia. A case study with bone marrow findings before and after splenectomy. *Tr. A. Am. Physicians* 59: 276-281, 1946.
- 3 DOAN, C. A. Neutropenic state. Its significance and therapeutic rationale. *J. A. M. A.* 99: 194-202, 1932.
- 4 EMBLETON, D. Rhythmical agranulocytosis. case report. *Brit. M. J.* 2: 1258, 1936.
- 5 IMERSLUND, O. A case of cyclic agranulocytosis. *Acta Paediat.* 30: 232-250, 1941.
- 6 THOMPSON, W. P. Observations on possible relationship between agranulocytosis and menstruation with further studies on a case of cyclical neutropenia. *New England J. Med.* 210: 176-178, 1934.
- 7 PLUM, P. Clinical and experimental investigations in agranulocytosis with special reference to the etiology. London: H. K. Lewis, 1937. p. 171.
- 8 MOORE, C. V., AND BIERBAUM, O. Chronic neutropenia treated by splenectomy. *Internat. Clin.* 3: 86-95, 1939.
- 9 CANTOR, M. M., AND SCOTT, J. W. Agranulocytic angina effectively treated with intravenous pyridoxine (vitamin B<sub>6</sub>). *Canad. M. A. J.* 52: 368-371, 1945.
- 10 FISHER, E. H., AND VORZIMER, J. Effect of pyridoxine on granulopenia caused by thiouracil. *Proc. Soc. Exper. Biol. & Med.* 60: 181-184, 1945.
- 11 MENTEN, M. L., AND GRAFF, E. Blood changes related to sulfonamide therapy. *Granulocytopenia associated with the use of sulfonamides.* *Am. J. M. Sc.* 221: 666-671, 1946.
- 12 WISEMAN, B. K., AND DOAN, C. A. A newly recognized granulopenic syndrome caused by excessive splenic leucolysis, successfully treated by splenectomy. *J. Clin. Invest.* 18: 473, 1939.
- 13 NORDENSON, N. G., AND RODEN, S. Chronic malignant granulocytopenia treated with splenectomy. Recovery. Report of a case. *Act. Chir. Scandinav.* 84: 519-526, 1941.
- 14 SALZER, M., RANSOHOFF, J. L., AND BLATT, H. Primary splenic neutropenia with report of a case. *Ann. Int. Med.* 22: 271-273, 1945.
- 15 ROGERS, H. M., AND HALL, B. E. Primary splenic neutropenia. *Arch. Int. Med.* 75: 100-106, 1945.
- 16 MUETHER, R. O., MOORE, L. T., STEWART, J. W., AND BROWN, G. O. Chronic granulocytopenia caused by excessive splenic lysis of granulocytes. Report of a case. *J. A. M. A.* 116: 255-257, 1944.
- 17 WISEMAN, B. K., AND DOAN, C. A. Primary splenic neutropenia. An entity related to congenital hemolytic icterus and essential thrombocytopenic purpura. *Ann. Int. Med.* 19: 1097-1117, 1942.

## CHRONIC NEUTROPENIA FAVORABLE RESPONSE FOLLOWING SPLENECTOMY

By LIEUT HARRY A. WEISS, (MC) U S N , AND LIEUT (JG) WILLIAM T. COLLINS,  
(MC) U S N R

THE ROLE of the spleen in the pathogenesis of neutropenia has been extensively studied in recent years, and two hypotheses have been advanced. Doan and his associates<sup>1</sup> have observed hyperplasia of the phagocytic reticulo-endothelial cells or clasmatocytes of the spleen with an abnormal phagocytosis of the granulocytes, and account for the neutropenia on this basis. They have also applied this concept of selective destruction of cellular elements of the blood to explain the anemia and thrombocytopenia which are frequently associated with the neutropenia.

The second hypothesis, which has been strongly supported by Dameshek,<sup>2,3,4</sup> is that of hypersplenism in which the spleen exerts an abnormal inhibitory effect, probably by means of a hormone, upon the maturation and release of cells from the bone marrow. Dameshek has emphasized this mechanism particularly in idiopathic thrombocytopenic purpura,<sup>2</sup> and also believes that the granulocytopenia which occurs in many types of splenomegaly may be mediated in a similar manner.<sup>3,4</sup>

A case of chronic neutropenia has been studied and is reported because of the significant elevation of the circulating neutrophils following splenectomy.

### CASE REPORT

V E, a 19 year old white male was admitted to the hospital on April 10, 1946 with a diagnosis of diabetes mellitus.

The patient's illness began in August, 1945, while aboard ship in the South Pacific, with symptoms of weakness, lassitude, somnolence, polydipsia, pronounced weight loss and muscular cramps in the legs. On September 20, 1945 he had a brief episode of generalized abdominal cramps and vomiting from which he rapidly recovered. A few days later, a second episode occurred and was accompanied by a mild diarrhea. Physical examination at that time was not remarkable but a blood count revealed leukopenia. Treatment consisted of paregoric and penicillin, and the cramps and the diarrhea ceased. Because of the persistence of the leukopenia, he was transferred to a Fleet Hospital in Manila, P. I. On admission there a physical examination revealed no significant findings. On November 4 a urinalysis disclosed a 4 plus sugar, and subsequently a glucose tolerance test showed a diabetic curve. The diabetes was controlled by insulin and dietary measures and the patient was transferred to a Naval Hospital in Hawaii for further study of the leukopenia. On January 16, 1946, a sternal biopsy was performed and revealed no evidence of blood dyscrasia. His course there was uneventful and on April 10, 1946 he was sent to this hospital.

The patient had been a lifelong resident of Utah until entry into the Navy in July, 1944. From 1937 to 1944, he had sprayed arsenic of lead insecticide in orchards for three to four day periods several times each summer. For one month prior to the onset of the present illness, he had worked in a paint locker on the ship six hours each day but, to his knowledge, had not handled any lead paints. From March to September, 1945, he had received atabrin in prophylactic dosage.

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From the U S Naval Hospital, Oakland, California

The opinions expressed herein are those of the authors and are not necessarily those of the Navy Department.

The patient's father, mother and ten siblings were all living and well. There had been no known occurrence of diabetes or blood dyscrasias in the family.

On admission, the patient had no complaints, and physical examination revealed a well developed well nourished, young white male with no positive findings. The urine contained 4 plus sugar and the fasting blood sugar was 315 mg. per cent. The leukocyte count was 2,650 per cu. mm. with 1 per cent bands, 56 per cent segmented forms and 43 per cent lymphocytes.

The patient was placed on a diet of 2300 calories and the insulin dosage was regulated at 50 units regular and 30 units of protamine zinc insulin mixed in the same syringe and given daily before breakfast. Therapeutic agents given in an attempt to correct the leukopenia included the following: pentnucleotide 10 cc. intramuscularly daily April 18 to May 13, refined liver extract, 0.1 cc. intramuscularly daily May 24 to June 1, crude liver extract 1.0 cc. intramuscularly daily, June 2 to July 1, liver broth 500 cc. orally daily May 4 to May 18. None of these agents had any appreciable effect upon the number of neutrophils in the circulating blood.

On May 22, an abscessed tooth was extracted, penicillin being used prophylactically for several days. Two examinations disclosed normal vision and ocular fundi. Neither the spleen or the liver were palpated at repeated examinations of the abdomen. Roentgenograms of the chest and of the flat and long bones revealed no abnormalities. Gastric analysis using 100 cc. of 7 per cent alcohol as a stimulant showed no free acid in the fasting, the 30 minute and the 45 minute specimens but 16 and 20 degrees of free acid were

TABLE 1

Hour	Leukocytes	Neutrophilic		Lymphocytes	Eosinophils	Monocytes
		Bands	Segs			
9 00 AM	2,900	2	25	65	4	3
9 10	Clamping of the splenic artery and vein					
9 15	4,300	3	31	61	2	3
9 30	4,500	10	47	40	3	
9 45	5,700	12	63	23	1	
10 15	8,700	14	55	31		
11 00	7,300	11	61	22	3	3
12 00	8,600	15	63	16		5
2 00 PM	10,400	19	64	13	2	2
6 00	12,150	12	72	14		2

present in the 60 and the 75 minute specimens respectively. The subcutaneous injection of 0.7 cc. of 1:1000 solution of epinephrine hydrochloride produced a maximum rise in the blood sugar from 64 mg. per cent in the fasting specimen to 121 mg. per cent in the 90 minute specimen.

During the last month prior to surgery the prothrombin time, the bleeding time and the clotting time were found to be normal.

The patient's course was uneventful until October 15, 1946, when a splenectomy was performed by Capt. Harold F. Young, MC, U. S. Navy. The convalescence was uneventful and the patient was discharged from the Navy on December 24, 1946, because of the diabetes mellitus.

### BLOOD FINDINGS

1. *Cellular elements* The erythrocyte and the hemoglobin determinations revealed no appreciable deviation from normal throughout the hospital course. The leukocyte and the neutrophil counts performed in this hospital are shown in figure 1.

On the day of surgery, leukocyte and differential counts at frequent intervals revealed an immediate increase in the number of circulating neutrophils (table 1).

By the afternoon of the first postoperative day the leukocytes had dropped to 5,650 per cu. mm. and fluctuated only slightly thereafter. On the day of discharge, December 24, 1946, the leukocyte count was

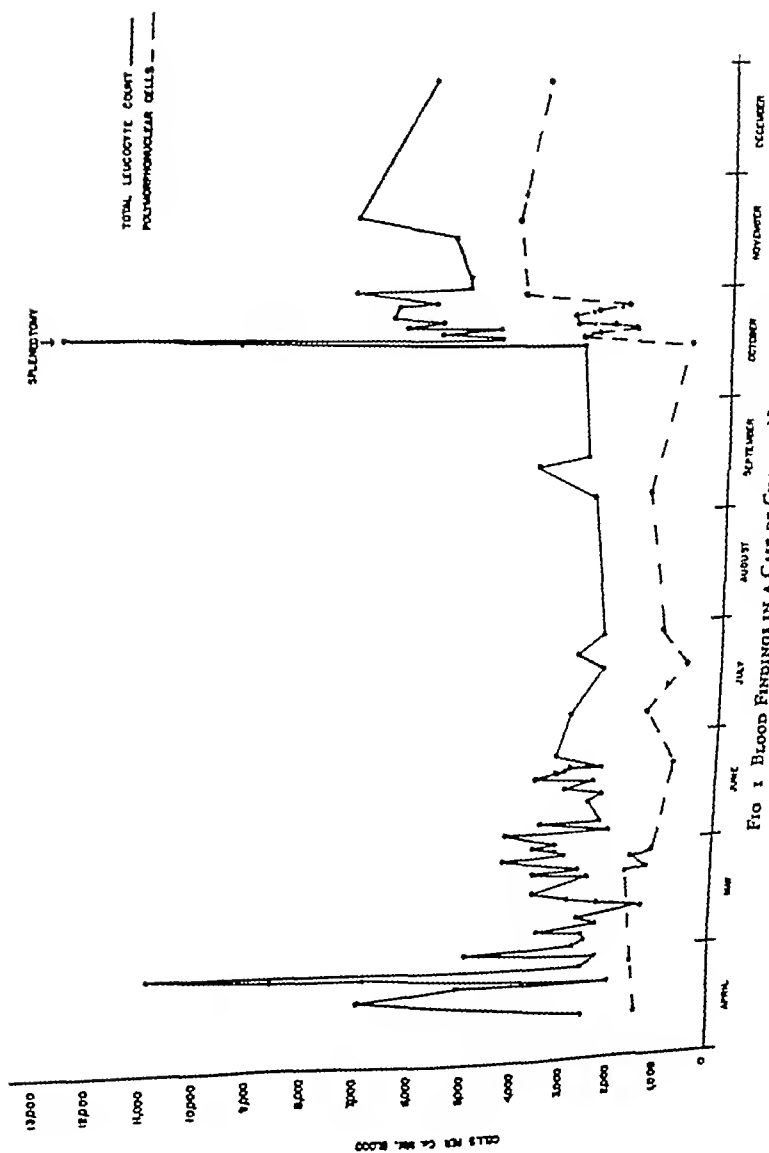


FIG 1 BLOOD FINDINGS IN A CASE OF CHRONIC NEUTROPENIA

5,850 with 63 per cent segmented neutrophils, 33 per cent lymphocytes, 2 per cent eosinophils, and 4 per cent monocytes

Through the courtesy of Dr. M. M. Wintrobe of Salt Lake City, Utah, where the patient is now residing, the following values were obtained on February 4, 1947: 6,050,000 erythrocytes per cu. mm., 19.0 grams hemoglobin per 100 cc. of blood, 4,500 leukocytes per cu. mm., with a differential of 42 per cent segmented neutrophils, 38 per cent lymphocytes, 6 per cent eosinophils, and 14 per cent monocytes.

## 2. Mean erythrocyte determinations

Date	RBC	Hb	PCV	MCV	MCH	MCHC
6-21-46	5.08	14.5	53.0%	104	28.5	27.4
7-18-46	5.20	16.0	52.5	101	30.7	30.4
9-12-46	4.72	13.5	50.0	106	28.6	27.0
2-4-47	6.05	19.0	55.2	91	31.0	34.0

3. *Platelets* Numerous counts done by the indirect method of Fonio revealed an average concentration of 250,000 per cubic millimeter both pre- and postoperatively.

4. *Erythrocyte fragility* Determination by Sanford's method on two occasions revealed no significant deviation in the fragility of the patient's erythrocytes from that of the control.

5. *Cellular response following the injection of adrenalin* This procedure was done five days prior to splenectomy in an effort to determine if the spleen was a significant reservoir of blood. The dosage of adrenalin was 1.0 cc. of a 1:1,000 solution administered by the subcutaneous route. Blood pressure and pulse were recorded to ascertain the time of maximum response.

Time	Pulse	Blood pressure	Leukocytes per cu. mm.	Platelets per cu. mm.
Before injection	60	118/65	2,850	200,000
15 minutes after injection	62	122/68	8,100	240,000
30 minutes after injection	68	135/70	12,200	255,000
45 minutes after injection	65	125/65	6,300	204,000

The same procedure was repeated approximately one month following splenectomy as a control measure.

Time	Pulse	Blood pressure	Leukocytes per cu. mm.	Platelets per cu. mm.
Before injection	68	124/64	7,500	270,000
15 minutes after injection	80	146/66	9,750	400,000
30 minutes after injection	76	136/55	9,250	300,000
45 minutes after injection	70	135/56	9,000	340,000

6. *Sternal marrow study* Aspiration of the sternal marrow was performed on July 19, 1946, and the findings were as follows: Myeloblasts 1 per cent, myelocytes and metamyelocytes 9 per cent, erythrocytic band forms 17 per cent, neutrophilic segmented forms 3 per cent, eosinophils 1 per cent, lymphocytes 10 per cent, no pronormoblasts, normoblasts and macronormoblasts 39 per cent. The myeloid-erythroid ratio was 1:3. The production of granulocytes appeared to be somewhat decreased. A very few macrophages were found in the smears as resting forms or naked nuclei.

\* We gratefully acknowledge the assistance rendered by Dr. Harry Wexler of San Francisco and Commander John S. Shaver, MC, U. S. Navy, in the interpretation of the histology of the bone marrow and the spleen.

## PATHOLOGY

The spleen weighed 230 grams and measured  $14 \times 9 \times 5$  centimeters. The capsule was thin and translucent. Sections revealed a slightly congested, firm, reddish-tan pulp in which the Malpighian corpuscles were readily visible.

*Microscopic examination* The capsule and trabeculae of the spleen were of normal thickness and consisted of dense connective tissue and a scattering of smooth muscle cells. Histologically, the chief findings consisted of an increase in number and size of the lymphoid follicles, particularly the germinal centers and a moderate hyperplasia of the reticulo-endothelial elements lining the dilated sinusoids with enlargement of the splenic or Billroth's cords. The pulp was fairly devoid of erythrocytes but contained a moderately increased number of leukocytes of the polymorphonuclear type. The sinusoids were dilated and contained stagnated white blood cells of the granulocytic series. Only an occasional macrophage was found which contained identifiable nuclear fragments of the granulocytic series and this was considered minimal or within normal limits after comparison with normal splenic tissue from similar age groups. Many of the lining sinusoidal endothelial cells were laden with coarse granular brownish-black pigment, and an occasional degenerated red blood cell. No phagocytized white blood cells were found in these cells. The hyperplastic lymphoid follicles were unevenly distributed throughout the parenchymal tissue, and the sheathed arteries were not remarkable. Impression smears and supravital stains were not made.

## SUMMARY AND CONCLUSIONS

The case of a patient with chronic neutropenia without splenomegaly, but responding favorably to splenectomy is reported. The surgical procedure appeared to be indicated by the following: (1) exclusion of the extrinsic causes of neutropenia, (2) failure of response to the agents commonly employed to stimulate granulopoiesis, (3) demonstration of granulopoiesis in the sternal marrow, (4) increase in the circulating neutrophils following the parenteral administration of epinephrine, (5) the presence of coexisting diabetes with the potential hazard of infection.

The implication of the spleen as the main factor in the causation of the neutropenia in this case seems well established, although the specific mechanism is not apparent. There was no evidence of abnormal phagocytosis in the microscopic examination of the spleen.

## ACKNOWLEDGMENT

We wish to express appreciation to Captain Earl F. Evans, Medical Corps, U. S. Navy, for his assistance in the management of this case.

## REFERENCES

- <sup>1</sup> DOAN, C. A. AND WRIGHT, C. S. Primary congenital and secondary acquired splenic panhematopenia. *Blood* 1: 10-26, 1946.
- <sup>2</sup> DAMESHEK, WILLIAM. The megakaryocytes in idiopathic thrombocytopenic purpura: a form of hypersplenism. *Blood* 1: 27-30, 1946.
- <sup>3</sup> ———. Editor's footnote. *Blood* 1: 12, 1946.
- <sup>4</sup> ———. Editorial. *Blood* 1: 173-174, 1946.

# LEUKEMOID REACTION DUE TO MIXED MALARIA INFECTION

## REPORT OF A CASE

By CAPTAIN JAMES A. RILEY, MC, AUS AND MAJOR GEORGE M. ROBINS, MC, AUS

**F**ALCIPARUM malaria has been rare in this country in returned soldiers and veterans because of the widespread use of atabrine as a suppressant in overseas areas. The suppressive dosages of this drug are believed to have been curative rather than merely suppressive for *P. falciparum* infections.<sup>1</sup> Recently at the Tilton General Hospital an instance of mixed malarial infection was seen in which both *P. vivax* and *P. falciparum* parasites were found in the peripheral blood smears (fig. 1). Another unusual feature in this case during the severe stage of the illness was a leukemoid picture in the peripheral blood and bone marrow. References to such reactions in the literature are scanty. Morin<sup>2</sup> reported a leukemoid reaction occurring in a young Greek woman who had relapsing malaria of eighteen months duration that had been inadequately treated before she came under his observation. He found the initial red blood cell and platelet counts to be normal and the white blood count to be 6,600 per cu. mm. with 73 per cent promyelocytes and 14.5 per cent myelocytes in the differential count. With quinine therapy the leukemoid blood picture returned to normal within two months. No bone marrow study was done. Schilling<sup>3</sup> states that acute tropical malaria may produce a marked shift to the left even to the extent of myelocytosis. Beregoff<sup>4</sup> found that malaria patients dying in coma showed low white blood cell counts and a marked shift to the left with neutropenia. Hill and Duncan<sup>5</sup> in their paper on leukemoid reactions refer briefly to the possibility of leukemoid reactions occurring in blackwater fever.

Because of the apparent rarity of leukemoid reactions in malaria and the infrequency of *falciparum* malaria in this country, the following case is reported.

## CASE REPORT

A 28 year old Negro male was admitted to Tilton General Hospital on Nov. 29, 1946 complaining of high fever, shaking chills, and profound weakness. He had been well until about November 1, 1946 when he was en route to the U. S. from Manila, Luzon, P. I. where he had been stationed for the previous nine months. On this date he developed chills, fever, headache, malaise, and weakness. Chills and fever occurred in paroxysms every other day for ten to fourteen days when all symptoms disappeared. The only treatment he received consisted of several large white pills of unknown composition each day. He arrived in the U. S. on November 18, 1946 and was sent from the debarkation point to the separation center and was home in Atlantic City by Nov. 22, 1946. On November 23, 1946 all his previous symptoms returned and again the chills and fever occurred approximately every second day. He was then admitted to the Tilton General Hospital. He stated that during his stay in the Philippine Islands he had never received any suppressive medication for malaria nor had he had malaria. As a child he had uncomplicated measles and chickenpox, and an appendectomy in 1934 with drainage from the wound for two weeks following surgery. The patient had lived in Richmond, Va. until 1934 when he moved to Atlantic City. There was no past history of episodes of chills, fever, or other illness. The family history was non-contributory.

On his admission the patient fairly well nourished and well developed, appeared acutely ill and exhibited evidence of weakness and weight loss. The rectal temperature was 96.4° F. A few tremors of the

From the Medical Service, Tilton General Hospital, Fort Dix, New Jersey.



upper limbs and head was present. All mucous membranes were quite pale. Examination of the eyes, ears, nose, mouth, and throat was negative. The ocular fundi were normal. The neck and thyroid gland were normal. No lymph nodes were palpably enlarged. The chest was symmetrical and the lungs were clear to auscultation and percussion. The pulse rate was 100 per minute and peripheral vessels were normal. The blood pressure was 110 systolic and 65 diastolic. The heart was not enlarged; the rhythm was regular, and a soft systolic apical murmur was present which was not transmitted and which varied somewhat with respiration. The abdomen was slightly distended and tympanitic, the liver and spleen were not palpably enlarged, but the splenic area was tender. Neurologic examination was normal except that the

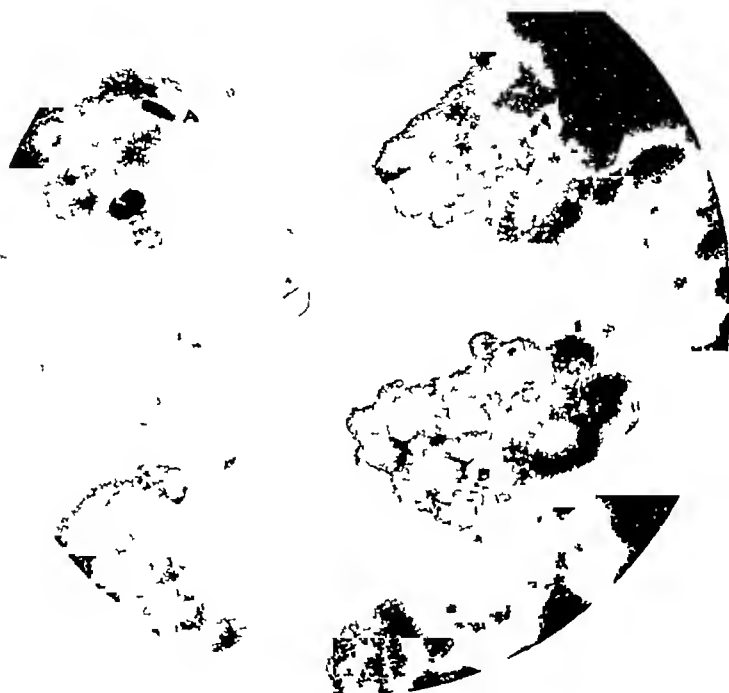


FIG. 1.—(A) GAMETOCYTE *PLASMODIUM FALCIPARUM* (B) TROPHOZOITE, *PLASMODIUM VIVAX*  
(THICK SMEAR PERIPHERAL BLOOD)

patient showed some confusion as to the sequence of recent events and some retardation of cerebration. No other abnormalities were noted.

**Laboratory examinations.** The red and white blood counts on admission and during the hospital course of the patient are tabulated (table 1). On admission both thick and thin blood smears were negative for malarial parasites. The urine showed a specific gravity of 1.010 with a trace of albumin, no sugar, and an occasional white blood cell per high power field on microscopic examination. Two blood cultures were negative on December 14, 1946, and on January 10, 1947. No red blood cell sickling was demonstrated; the test being read at intervals up to twenty-four hours. Urinalysis on January 8, 1947, showed a specific gravity of 1.015, albumin and sugar negative, and 8-10 white blood cells, occasional granular

TABLE I—Blood Counts

	Nov 29 46	Dec 3 46	Dec 9 46	Dec 16 46	Dec 19 46	Dec 31 46	Jan 6 47	Jan 11 47	Jan 21 47	Feb 3 47
Red blood count in mil lions per cc.	1 16	3 06	2 37	2 4	—	—	3 07	—	3 25	—
Hemoglobin in Gm %	4 0	6 0	7 0	8 0	9 0	6 0	8 5	—	10 5	—
White blood count	4000	—	8450	5350	4450	8400	6150	5000	5900	4700
Neutrophils %	27	—	67	34	25	69	—	39	47	62
Eosinophils %	0	—	0	—	2	1	—	1	1	4
Band form cells %	14	—	0	30	14	0	—	18	21	4
Metamyelocytes %	21	—	0	0	0	0	—	0	0	0
Myelocytes %	11	—	0	0	0	0	—	0	0	0
Lymphocytes %	21	—	33	32	55	26	—	39	26	29
Monocytes %	6	—	0	4	4	4	—	3	5	1
Platelet count in thou sands	—	—	120	—	—	—	—	—	—	—

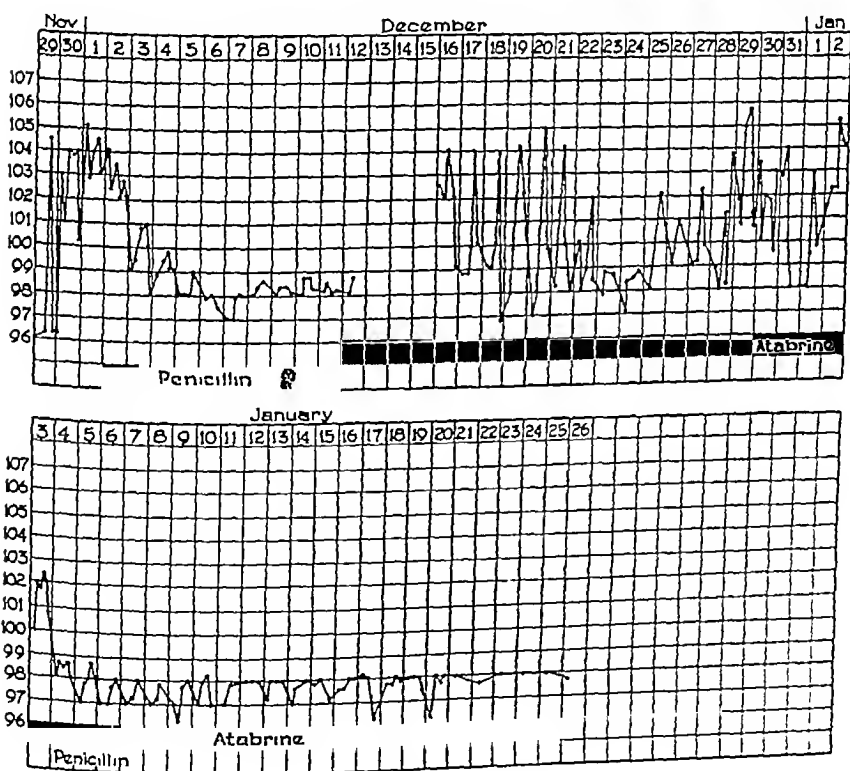


FIG 2.—GRAPHIC TEMPERATURE CHART

casts and 0-2 red blood cells per high power field. A test for bile pigment was negative. Leucocytes were present in the urine in normal amounts.

On the patient's admission a diagnosis of malaria was made. However, this seemed improbable when two smears were negative for malarial parasites and the admission white blood count suggested aleukemic myelogenous leukemia. Therefore the patient was transfused with 500 cc of whole blood on November 30, 1946 and penicillin was given in 40,000 unit doses intramuscularly every three hours. Five hundred cubic centimeter blood transfusions were repeated on December 1, 1946, December 3, 1946 and January 5, 1947. On this therapy, together with 5 per cent glucose in saline intravenously, the patient's fever gradually receded and reached normal on December 3, 1946 (fig. 2). From December 6 to 15, 1946, the patient was afebrile and, except for weakness and malaise, free of symptoms.

On December 4, 1946, in an effort to clarify the blood picture and confirm the diagnosis of leukemia, a sternal bone marrow aspiration was done. The results of this and of a later bone marrow study are outlined in Table 2.

TABLE 2.—*Differential Counts of Sternal Bone Marrow Aspirations*

	Dec. 4 '46	Feb. 24 '47
%		
Neutrophils	3.6	18.0
Stab cells	0.0	3.2
Lymphocytes	8.4	19.2
Monocytes	1.2	4.2
Eosinophils	1.0	0.0
Basophils	0.0	0.0
Metamyelocytes		
Neutrophilic	11.5	5.8
Eosinophilic	0.9	2.4
Basophilic	0.0	0.6
Myelocytes		
Neutrophilic	18.8	8.6
Eosinophilic	0.9	2.4
Basophilic	0.0	0.2
Premyelocytes	21.3	3.8
Myeloblasts	7.9	1.2
Megakaryocytes	0.5	1.4
Erythroblasts	7.8	3.4
Normoblasts	12.4	19.6
Unidentified	3.8	1.2
Degenerated	0.0	4.8

The bone marrow study of December 4, 1946 showed a marked increase in the early forms of the neutrophilic series. Although the blast forms were not markedly increased, there was an increase in promyelocytes and myelocytes. Many degenerated cells were seen which were believed to be degenerating myelocytes and metamyelocytes. The smear was considered compatible with myelogenous leukemia.

After eight afebrile days the patient again began to have fever on December 16, 1946. The course of the temperature from then on is illustrated in figure 2. Each rise in temperature was accompanied by severe chill, marked headache and myalgia. On December 15, 1946 the physical findings were unchanged from those noted previously except that the spleen was palpable and quite tender. A blood smear taken on December 28, 1946, was found positive for *P. falciparum* and *P. vivax* (fig. 1). On December 30, 1946 treatment was started with atabrine in doses of 0.2 grams every six hours for 5 doses, then 0.1 gram three times a day for six days, and then 0.1 gram daily for the next thirty days. By January 4, 1947 the temperature was normal and the patient was greatly improved. From then on his convalescence was uncomplicated.

The previous bone marrow and peripheral blood smears were re-examined after the correct diagnosis

had been made, but again no malarial parasites were found. The final bone marrow study done on February 24, 1947, was normal. The severe anemia and the leukemoid reaction had disappeared.

The patient was discharged from the hospital on April 8, 1947, in good health. He was still well one month later.

### COMMENT

While it has been previously reported that immature cells of the granulocyte series occasionally appear in the peripheral blood during paroxysms of malaria, there is only one previous case report in the literature showing a marked shift to the left of the granulocyte series. No cases have been found in which bone marrow biopsies have been performed.

Because of the difficulties of diagnosis posed by a leukemoid picture in malaria and the rarity of this finding, this case is reported. It is possible that the increased hemolysis and the attending anemia are responsible in certain cases for sufficient bone marrow stimulation to cause this change.

### SUMMARY

A case of mixed malarial infection with a leukemoid blood picture is reported. The literature is reviewed.

### REFERENCES

- <sup>1</sup>WAR DEPARTMENT Technical Bulletin No. 65, July 1944. WAR DEPARTMENT Technical Bulletin No. 182, July 1945.
- <sup>2</sup>MORIN, H. G. S. Myélocémie dissocée transitoire chez une paludéenne. Bull. et mem. Soc. med. d. hop. de Paris 49: 1419, 1915.
- <sup>3</sup>SCHILLING, V. The Blood Picture and Its Clinical Significance. R. B. H. Gradwohl translation, eds. 7 and 8. St. Louis, C. V. Mosby, 1929, p. 277.
- <sup>4</sup>BEREGOFF, PAULINE. The Schilling differential blood count in tropical disease. J. Lab. & Clin. Med. 19: 644, 1933-34.
- <sup>5</sup>HILL, J. M. AND DUNCAN, C. N. Leukemoid reactions. Am. J. M. Sc. 201: 847-857, 1941.

# ABSTRACTS

JOSEPH F ROSE M.D. Editor

## ABSTRACTERS

HELEN W BELDING, M.D. Winston Salem N C	OLIVER P JONES Ph D Buffalo
SHEILA T CALLENDER M.D. Oxford England	CONRAD MAIER, M.D. Zurich Switzerland
GEORGE E CARTWRIGHT M.D. Salt Lake City	MILOŠ NETOUŠEK, M.D. Prague, Czechoslovakia
ROGER C CRAFT, Ph D Boston	JEAN P SOULIER, M.D., Paris
CHARLES P EMERSON M.D., Boston	RAMON M SUÁREZ San Juan Puerto Rico
SOLOMON ESTREN M.D. New York	WILLIAM VALENTINE M.D. Los Angeles
CLEMENT A FINCH M.D. Boston	JAN WALDENSTRÖM, M.D., Upsala Sweden

## ERYTHROCYTES AND ERYTHROCYTIC DISEASE

ACTIVITY OF MICROBIAL ANIMAL PROTEIN FACTOR CONCENTRATES IN PERNICIOUS ANEMIA *E L R Stokstad A Page J Pierce A L Franklin T H Jukes R M Henle W Epstein and A D Welch* From the Lederle Laboratories Division American Cyanamid Company Pearl River, New York and the Departments of Medicine and Pharmacology, School of Medicine, Western Reserve University Cleveland Ohio *J Lab & Clin Med* 33 860-864 1948

These investigators have found that a nonmotile rod-shaped organism from hen feces when grown aerobically on simplified media produces appreciable quantities of the animal protein factor as indicated by assay with chicks on diets deficient in this factor. Since it is known that refined liver extracts produce a growth response in chicks deficient in the animal protein factor concentrates of the microbial animal protein factor were tested for anti pernicious anemia activity in two patients with pernicious anemia in relapse. The results indicate that the concentrates were active in inducing a hematopoietic response in such patients. Whether the active substance in the microbial concentrates is identical with the anti pernicious anemia factor or the recently isolated vitamin B<sub>12</sub> is not known. The answer to this will have to await chemical identification of the various substances. The possibility exists that factor X, the cow manure factor zoopherin the animal protein factor, the microbial animal protein factor vitamin B<sub>12</sub> and the anti pernicious anemia substances are all similar, identical or related compounds.

G E.C.

PRESENCE OF COBALT IN THE ANTI PERNICIOUS ANAEMIA FACTOR *E L Smith* From Glaxo Laboratories Ltd Greenford Middlesex England *Nature London* 162 144 1948

Examination of crystals of anti pernicious anemia factor has shown the presence of cobalt. If each molecule contains one atom of cobalt the molecular weight of the compound is about 1500. Allowance for loss on drying brings this in excellent agreement with that found on x ray crystallography (1550-1750). The higher value (3000) found by the diffusion method may be due to errors inherent in the method, the use of impure material in earlier experiments, or that association occurs in solution. Analytic figures indicate that the molecule contains three atoms of phosphorous. Reference is made to the fact that the Merck workers have found cobalt and phosphorous in their vitamin B<sub>12</sub>.

S T.C.

CONCENTRATION OF COBALT BY MICRO-ORGANISMS AND ITS RELATION TO COBALT DEFICIENCY IN SHEEP *J Tossie and Mitchell* From Rowett Research Institute, Bucksburn Aberdeenshire and Macaulay Institute for Soil Research Craigiebuckler Aberdeen Scotland *Nature London* 162 502-504 1948

Reference is made to previous work on cobalt as an essential factor in ruminant nutrition. Observations on three sheep with rumen fistulae are described. Sheep A was given a seeds hay diet (0.27 p.p.m. cobalt in dry matter). Sheep B and C bred originally on a cobalt deficient pasture were maintained on a hay diet containing only 0.07 p.p.m. cobalt. C was given 1 mg. added cobalt daily. After 6 weeks rumen contents were collected, fractionated, examined microscopically and for cobalt content. The data make it apparent that rumen micro-organisms concentrate cobalt from their external environment and that the cobalt concentration of the microbial population is related to cobalt content of the diet.

It is suggested that absorption of cobalt by the host may deprive micro-organisms of an essential factor, the host in turn being deprived of essential bacterial products, or if cobalt is an essential metabolite for the host alone, concentration in the micro-organisms may reduce its availability. Again host and alimentary micro-organisms may both require cobalt for metabolic activities, the competition being important on cobalt deficient diets.

Although this work is superficially remote from hematology the finding of cobalt in the crystalline anti-pernicious anemia factor indicates that it may be important in helping to elucidate the problem of megaloblastic anemias, especially those of intestinal origin. It may also link up with the experimental macrocytic anemia in rats (Watson et al. *Lancet* 2, 404, 1948) in which a gross change of microbial population of the intestine is probably produced.

STC

THE TREATMENT OF SUBACUTE COMBINED DEGENERATION OF THE SPINAL CORD WITH VITAMIN B<sub>12</sub>. T. D. Spies, R. E. Stone, S. Kartus and T. Aramborn. From the Department of Nutrition and Metabolism of Northwestern University at the Hillman Hospital, Birmingham, Alabama. *South M. J.* 41, 1030-1031, 1948.

The response to crystalline vitamin B<sub>12</sub> of 3 patients with pernicious anemia exhibiting acute neurologic manifestations is reported briefly. No data is presented other than that included in the one representative case report. The case is that of a 48 year old male with pernicious anemia who lapsed to treatment and presented a three weeks history of glossitis, weakness and inability to walk unsupported. Blood studies revealed a moderate slightly macrocytic anemia. Four parenteral injections of 25 micrograms each of vitamin B<sub>12</sub> were given at forty-eight hour intervals. Within the first forty-eight hours pain and tenderness of the legs and soreness of the tongue had disappeared and by the fourth day the patient could walk without support. Improvement in although not disappearance of abnormal neurologic signs (other than the return of a normal plantar response) was noted on the fifth day. A reticulocytosis of 14 per cent occurred on the fifth day followed by elevation of the red cells, white cells, platelets and hemoglobin (no figures given). Reference is made to the relief from neurologic symptoms noted in three previously reported cases following a single injection of 25 micrograms of vitamin B<sub>12</sub> (Postgrad M. 4, 89-95, 1948).

As the authors admit, insufficient time has elapsed to evaluate the effectiveness of vitamin B<sub>12</sub> as maintenance therapy for patients with pernicious anemia with or without neurologic complications. Certainly the experience with folic acid has shown that one must be extremely cautious in drawing conclusions from an initial neurologic improvement of several days.

H W B

CRYSTALLINE ANTI-PERNICIOUS-ANEMIA FACTOR IN TREATMENT OF TWO CASES OF TROPICAL MACROCYTIC ANEMIA. J. C. Patel. From Singhanee Hindu Hospital, Bombay, India. *Brit. M. J.* 2, 934-935, 1948.

Two cases of tropical macrocytic anemia occurring in Bombay showed a good response to single injections of 80 µg. each of Lester Smith's crystalline anti-pernicious anemia factor. This appears to be an interesting observation in view of previous suggestions that such cases are deficient in 'Wall's factor' rather than the anti-pernicious anemia factor.

STC

EXPERIMENTAL MACROCYTIC ANAEMIA IN THE RAT. G. M. Watson, D. G. Cameron and L. J. Wiles. From the Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Oxford, England. *Lancet* 2, 444, 1948.

Anemia resembling pernicious anemia sometimes develops in association with intestinal disease. This suggested a method for producing macrocytic anemia in rats. Two operations were devised. In the first

A the intestine was divided the lower part tied off and the upper part was anastomosed to the gut some 12 inches below the blind end. One hundred and eighteen rats survived operation and 21 developed macrocytic anemia six weeks to one year later. As anemia followed only when there was some stenosis at the anastomosis or dilatation of the loop operation B was devised. Here the lower part was anastomosed to the gut 12 inches above the division later modified to 3 inches. Peristalsis then filled the loop which always became dilated. Seventeen of 24 rats survived the 3 inch operation and 13 developed progressive anemia after an average of eight weeks.

The anemia is normo- or hyperchromic with an increase in red cell diameter and irregular reticulocytosis. The bone marrow shows an increase in proerythroblasts and basophil erythroblasts. Treatment with liver extract (total of 0.1 to 0.8 ml. Anahaemin) suggests that the anemia responds unless there are complicating infections. One rat injected with 15 mg. of folic acid also showed a good response. Further work is in progress to determine how specific are these responses to treatment.

Such experiments may provide the long sought test animal for the therapeutic potency of liver extracts although further advances in the research on crystalline anti-pernicious anemia factor may render this unnecessary. More important is the fact that the technique opens up a new field for the investigation of the pathogenesis of macrocytic anemia.

S.T.C.

**THE LIFE SPAN OF THE MEGALOCYTE AND THE HEMOLYTIC SYNDROME OF PERNICIOUS ANEMIA.** K. Singer, J. C. King and S. Robin. From the Department of Hematologic Research and the Department of Pediatric Research, Medical Research Institute, Michael Reese Hospital, Chicago, Illinois. *J. Lab. & Clin. Med.* 33: 1068-1076, 1948.

Determinations of the average life span of red cells from 4 patients with pernicious anemia in relapse were performed using the method of differential agglutination (Ashby technique). It was found that the survival time of the red cells when injected into normal individuals was markedly decreased (27 to 75 days). After adequate treatment, the life span of the cells from the pernicious anemia patients became normal. These observations, as the authors conclude, are evidence for the concept that pernicious anemia is a true hemolytic syndrome caused by an intracorporeal mechanism. The shortened life span of the cells would seem to account for the increased pigment production observed in this disease but it is difficult to correlate with the observations of London, Shemin and Rittenberg using labeled glycine which indicate that a considerable portion of the pigment production is derived from sources other than hemoglobin. It is likely that this problem is somewhat more complicated than it appears to be.

G.E.C.

**THE LIFE SPAN OF THE SICKLE CELL AND THE PATHOGENESIS OF SICKLE CELL ANEMIA.** K. Singer, S. Robin, J. C. King and R. N. Jefferson. From the Department of Hematologic Research and the Department of Pediatric Research, Medical Research Institute, Michael Reese Hospital and the Department of Pediatrics, Provident Hospital, Chicago, Illinois. *J. Lab. & Clin. Med.* 33: 975-984, 1948.

This paper deals with cross determinations of the survival time of sickle cells. Trait cells were transfused into patients with sickle cell anemia and anemia cells into healthy recipients displaying the sickle cell trait. It was found that the trait cells survived normally when transfused into patients with sickle cell anemia whereas the patient's own cells continued to be hemolyzed at a faster rate. Cells from patients with sickle cell anemia when transfused into trait carriers had a shortened life span with an average of about one fourth of the normal. Therefore the pathogenic principle operating in sickle cell anemia would appear to reside within the red cells themselves rather than in an extracorporeal mechanism. The authors conclude that the sickling process is by itself not a satisfactory explanation of the pathogenesis of the anemia. They speculate that sickle cell anemia develops because of an additional alteration in the cytoskeleton which is qualitatively different from the structural anomaly responsible for the sickling phenomenon.

G.E.C.

**A SIMPLE AND RAPID METHOD FOR DEMONSTRATING SICKLING OF THE RED BLOOD CELLS. THE USE OF REDUCING AGENTS.** G. A. Daland and M. B. Castle. From the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital and the Department of Medicine, Harvard Medical School, Boston, Mass. *J. Lab. & Clin. Med.* 33: 1081-1088, 1948.

A simple and rapid method of producing sickling of the red blood cells in wet cover slip preparations of the blood of patients with sickle cell anemia is described. The principle on which the test is based is the production of reduced hemoglobin in the red cells by the addition of a reducing agent. In order to perform the test, a drop of a five fold aqueous dilution of Cevalin (approximateing a 2 per cent solution of buffered ascorbic acid and also containing 0.1 per cent sodium bisulfite) or a drop of 2 per cent sodium bisulfite  $\text{Na}_2\text{SO}_3$  is added to a small drop of the patient's blood on a glass microscope slide. After mixing, a cover slip is dropped on the preparation and excess blood is expressed by gentle pressure in order to produce a film of blood sufficiently thin to permit inspection of individual red cells under the high power objective of the microscope. With the diluted Cevalin solution, sickling of the blood usually appeared within an hour, and with the 2 per cent bisulfite solution it was often present within fifteen minutes at room temperature.

G. E. C.

**DETERMINATION OF HAEMOGLOBIN V. PRECISION OF COLORIMETRIC METHODS** E. J. King, M. Gilchrist, J. D. P. Weston. From Postgraduate Medical School, London. R. Donaldson, R. B. Sisson. From the National Physical Laboratory, Teddington. R. G. Macfarlane, H. M. Jope, J. R. O'Brien. From the Radcliffe Infirmary, Oxford. J. M. Pitterson, D. H. Strangeways. From the University of Wales, Cardiff. *Lancet* 2, 563-566, 1948.

On blood samples from 11 male and 10 female subjects hemoglobin determinations were made by means of iron analyses, oxygen and carbon monoxide capacities. Various hemoglobin derivatives were tested by several types of photometer, photoelectric colorimeter and in one series of experiments with a Hilger medium quartz spectrograph. For visual instruments, the true reading was taken as the mean of 20-50 readings obtained by several observers. The readings were compared with the base line values obtained from the iron analyses and gasometric determinations respectively. Analysis of the results showed that the neutral grey wedge photometer (King, E. J., *Biochem. J.* 41, Suppl. 32, 1947) compared favorably with the standard photoelectric and visual instruments. The single cell photoelectric colorimeter proved more reliable than the two celled absorptiometer. Of the hemoglobin derivatives oxyhemoglobin and cyanhematin gave the least variable results.

Most hematologic laboratories now use photoelectric colorimeters for hemoglobin estimation but where such instruments are not available the grey wedge photometer seems to provide a simple and accurate instrument.

S. T. C.

**THE NATURE OF THE ANEMIA OF PREGNANCY IN THE RAT** C. F. Bond. From the Department of Zoology, Cornell University, Ithaca, New York. *Endocrinology* 43, 180-186, 1948.

The purpose of this work was to determine the nature of the anemia which accompanies pregnancy in the adult female rat of the Long Evans strain. Calculations were made on the total erythrocyte count, hematocrit, hemoglobin level, whole blood and plasma specific gravities and blood volume. Studies were made at three stages of pregnancy and on the second day postpartum. A significant decrease in whole blood and plasma specific gravities occurred during pregnancy, but the total blood volume increased. Calculations of the total circulating erythrocytes and hemoglobin in pregnant rats showed a sharp rise in both elements. The author concludes from this evidence that the anemia which accompanies pregnancy in the rat is due to a hemodilution.

R. C. C.

**ON THE GENESIS OF MEGALOBLASTIC BLOOD FORMATION** U. Haerfel. Medizin. Abteilung des Stadt-Krankenhauses Mannheim (Germany). *Klin. Wschr.* 1948, 8-12.

Using supravital examinations of bone marrow in pernicious anemia the author advances the theory of origin of megaloblasts from undifferentiated reticulum cells. He considers megaloblastic blood formation as a mesenchymatous process in contrast to the normal parenchymatous erythropoiesis. Furthermore, he points out the necessity of accepting a second factor in the genesis of megaloblasts. He considers this factor to be the loss of the mitotic heteroplastic properties of the cells and assumes this to be the effect of the absence of the antipernicious principle.

The peculiar megaloblastic nuclear structure is considered as the persistence of mesenchymatous nuclear properties.

C. V.



**LAWFUL MITOTIC STRUCTURE IN CYTOPLASM OF MATURING BLOOD CELLS** *H Wenderoth* I Medizin Universitätsklinik, Hamburg Eppendorf (Deutschland) *Klin Wschr* 1948 182-183

Studying the mitosis of megaloblasts gained from the bone marrow in pernicious anemia the author observed a singular granular structure of the nucleus, which he was able to stain with the Giemsa method and which is called paramitotic granulation because of its temporary appearance during mitosis. This granulation has been formerly described by Rohr (and others) who however, thought it identical with the basophilic stippling of the erythrocytes. The author believes that the phenomenon has to be differentiated from the former. Apart from the megaloblasts, the paramitotic granulation was also observed in normal maturing erythrocytes and leukocytes.

The author believes that the granulation belongs to other mesenchymal elements as well. The described structures are looked on as mitotic-formed agglomerations of the basophilic substance. A relation to the basophilic stippling of the erythrocytes is probable.

C.M.

**TOXIC DAMAGE TO ERYTHROCYTES FINDINGS WITH ELECTRON-OPTIC INVESTIGATIONS OF ERYTHROCYTES** *F Jung* *Klin Wschr* 1947, 459

Based on his electron-optic studies this author deduces that erythrocytes possess a genuine membrane made of protein and containing hemoglobin inside. The membrane is covered on its surface by a lipid layer. The inside is formed by a spongelike stroma made of protein. This latter is easily denatured, causing a change in permeability properties and leading to hemolysis. The author studied the influence of different salts and of hemolytic substances. He considers the Heinz bodies a sign of degeneration. They are composed of coagulated parts of the cells which are more easily stained and not really located in the inside of the cells. They have no relation to methemoglobin.

C.M.

## THROMBOEMBOLIC DISEASE

**THE EARLY RECOGNITION OF POST-OPERATIVE VENOUS THROMBOSIS INCREASED PROTHROMBIN ACTIVITY AS AN AID TO DIAGNOSIS** *E B Mahoney and R S Sandrock* From the Department of Surgery of the University of Rochester School of Medicine and Dentistry and Surgical Service of Strong Memorial and Rochester Municipal Hospitals Rochester New York. *Bull New York Acad Med* 24 636-650 1948

The prothrombin activity using Quick's one stage method was determined preoperatively and followed daily through the sixth postoperative day in 68 patients, most of whom were considered likely candidates for venous thrombosis. In nearly all 58 postoperative patients who did not develop thrombosis there was a progressive decrease in prothrombin activity during the first three days followed by a gradual return to normal about the sixth day. All of the 10 patients who developed thrombosis on the other hand showed a rise to above normal on either the second or third day. This hyperprothrombinemia was interpreted as evidence of impending thrombosis although the prothrombin activity was usually normal by the time thrombosis was clinically evident. No satisfactory explanation was offered for the fact that the hyperprothrombinemia was more uniformly apparent in the undiluted than in the diluted plasma determinations. The authors suggest this test as a practical method of early detection of postoperative thrombosis and as a basis for selection of patients to receive prophylactic anticoagulant therapy.

The pathogenesis, diagnosis and prevention of thrombo-embolism are discussed. In their comparison of the relative merits of prophylactic vein ligation and anticoagulants the authors present more convincing evidence for the latter.

The whole problem of the relation of prothrombin activity to the occurrence of intravascular thrombosis has yet to be defined although there are fewer conflicting reports on changes in prothrombin activity in postoperative than in nonsurgical patients (coronary thrombosis etc.) who develop thrombosis.

H.W.B.

**THE QUESTIONABLE IMPORTANCE OF BLOOD CHANGES IN CORONARY OCCLUSION** *J H B Hilsm W M Cameron E S Mills and S R Townsend* From the Departments of Medicine and Haematology Montreal General Hospital Montreal Quebec Canada. *Canad M A J* 59 447-452, 1948

It was concluded from a study of 31 cases of acute coronary occlusion that there were (1) no constant

changes in blood coagulability as measured by the Waugh Ruddick test (prothrombin time (diluted and undiluted plasma) or coagulation time, (2) no constant changes in the Waugh Ruddick test during prolonged bed rest, (3) no constant variation in plasma protein levels during convalescence (4) a high percentage of patients with prolonged circulation times due to shock and/or myocardial weakness and (5) that the variance in blood volume studies depended on the presence or absence of shock and/or cardiac failure

This study is of interest because of the present controversial issue of whether or not there is a current increase in clotting tendency to account for the appreciable incidence of thrombo-embolism in myocardial infarction. The prothrombin studies support the work of Cotlove and Vorzimer (*Ann Int Med* 24 648, 1946) but are at variance with that of others e.g., Peters et al (*J A M A* 130 398 1946). The findings of normal or decreased blood coagulability by the Waugh Ruddick test in 77.4 per cent of cases on admission with a low incidence (0.14 per cent) of increased clotting tendency during hospitalization in half of the group which did not receive dicumarol varies considerably from the results obtained by Ogura et al (*J Clin Investigation* 25 586 1946).

The answer to this problem awaits a more complete understanding of the clotting mechanism and its relation to intravascular thrombosis as well as a greater refinement and uniformity in our laboratory tests. However convincing evidence is accumulating in large series of cases to justify the cautious prophylactic use of dicumarol in patients with coronary occlusion.

H W B

**EFFECT OF HEPARIN AND DICUMAROL ON SLUDGE FORMATION** *H Lausman, W B Marin and C Tarter*  
From the Department of Surgery Northwestern University Medical School Chicago Illinois  
*Science* 108 283-284, 1948

The mesenteric vessels of dogs were studied using the Kniseley technique. Sludging was produced by venous occlusion. Dogs were divided into four groups. Group 1 Control dogs. After venous occlusion sludge formation developed followed by adherence of sludged masses to the endothelium of the vessels. This was followed by the piling up of more cells to the agglutinated mass until the vessel was finally completely occluded. The thrombosis remained in many vessels after the venous circulation had been released. Group 2 Heparin was given after the appearance of sludge formation. Group 3 Heparin was given before venous occlusion. Group 4 Dicumarol was given before occlusion. In the last three groups although sludge formation did occur no thrombosis developed in any animal.

R C C

**VENOUS THROMBOSIS AND PULMONARY EMBOLISM** *A W Allen and G A Donaldson*  
From the Surgical Service of the Massachusetts General Hospital, Boston Massachusetts *Bull New York Acad Med* 24 619-635, 1948

The specific prophylactic or therapeutic measures used in 2,600 postoperative patients some of whom received a combination of methods are evaluated. With few exceptions these were patients over the age of 30. Prophylactic treatment by small doses of dicumarol was given to 496 patients. None of these died of pulmonary embolism although there were two fatalities associated with hemorrhage. Four of 871 patients who had prophylactic bilateral superficial femoral vein interruption died subsequently of pulmonary embolism whereas in this particular group of patients deemed likely to develop thrombosis 37 deaths from embolism might have been expected had specific measures not been used. Treatment for 1,266 patients was by phlebotomy, thrombectomy and femoral vein interruptions after clinical evidence of thrombosis occurred. There were six deaths in this group from further emboli compared to an estimated sixty had therapy been withheld.

This paper includes a good discussion of the factors predisposing to venous thrombosis. Emphasis is laid on the fact that despite the progress made since the advent of specific measures in the prevention and treatment of thrombo-embolism a statistically significant percentage of patients still die from massive pulmonary embolism.

The authors do not share the general enthusiasm of many for the prophylactic use of dicumarol in postoperative patients. They stress its hazards and condemn its empiric use without adequate clinical and laboratory control. It is their opinion that vein ligation is the safer and more effective method in the older debilitated or very ill patient.

H W B

## HEMORRHAGIC DISEASE AND BLOOD COAGULATION

THROMBOGENIC PURPURA, THE FAILURE OF DIRECT BLOOD TRANSFUSION TO RAISE THE PLATELET LEVEL.

*J S Lawrence W N Valentini, and W S Adams* From the Departments of Medicine and Radiation Biology, The University of Rochester School of Medicine and Dentistry, Rochester New York. *J Lab & Clin Med* 33 1077-1081 1948

This work was undertaken with the purpose of determining if massive direct transfusions of blood given to patients with thrombopenia purpura would raise the level of circulating platelets significantly. A patient with aplastic anemia was given 1500 ml. of whole blood within a short period of time. A second patient was given approximately the same amount of blood on two different occasions. Theoretically this amount of blood should have raised the circulating platelet levels about 100 000 per cu. mm. However, in only one of the three experiments reported was a significant increase noted in the recipient, and in this case it was so small as to be of little practical importance. The reasons for the unsatisfactory results are not evident, but the results suggest that either the life span of platelets is exceedingly short or that they are unusually rapidly utilized in thrombocytopenic conditions.

G.E.C.

SURGERY IN HAEMOPHILIA. A CASE OF SPINAL SUBDURAL HAEMATOMA PRODUCING PARAPLEGIA.

*Schiller G Nilgün and O Budtz-Olsen* From the Nuffield Department of Surgery the Children's Department, and the Department of Pathology, Radcliffe Infirmary, Oxford. *England Lancet* 2 842-845 1948

A haemophilic boy of 16 months developed complete paralysis of both legs and retention of urine. Cisternal myelogram showed complete arrest of the opaque medium at T9. Laminectomy was performed and a large subdural clot removed successfully. Convalescence was complicated by secondary haemorrhage from the wound but general progress was excellent and a high degree of functional recovery took place. Throughout the course in hospital the prolonged clotting time was controlled by repeated small transfusions of fresh blood (62 in the 66 days in hospital). Larger volumes of blood were used only to replace blood lost.

This case illustrates a rare complication of haemophilia and also shows clearly that major surgery may be safely undertaken with adequate control by transfusion.

S.C.

STUDIES ON A PROTEOLYTIC ENZYME IN HUMAN PLASMA. III. SOME FACTORS CONTROLLING THE RATE OF FIBRINOLYSIS.

*O D Ratnoff* From the Department of Medicine The Johns Hopkins University School of Medicine, Baltimore Maryland. *J Exper Med* 88 401-416, 1948

The phenomenon of fibrinolysis has long been recognized and it is now well established that the blood contains both a proteolytic enzyme system and inhibitors of this system. However, the factors responsible for rapid clot dissolution under certain conditions remain a subject of controversy.

The author in this well controlled in vitro study of factors governing clot lysis, has observed certain interesting phenomena. Caseinolysis was used as a measure of fibrinolytic activity and his methods for the determination of proteolytic and inhibitory activity of plasma are described in detail. He was unable to demonstrate that there was any correlation between the clot lysis time of recalcified plasma clots and the amount of proteolytic activity either spontaneously developed or activated by chloroform or streptococcal filtrate in a globulin precipitated from the same plasma. Furthermore, a constant relationship between the inhibitory activity of fresh plasma serum or albumin against plasma proteolytic enzyme and clot lysis time could not be shown. Following the discovery that this inhibitory activity was unstable and decreased during incubation however, it was possible to correlate clot lysis time with the deterioration of inhibitory activity occurring during incubation of recalcified plasma at 37°C. This inhibitory activity decreased until a minimal stationary level was reached and fibrinolysis occurred. The nature of the labile component of the inhibitory activity of plasma is now under investigation.

The fundamental importance of the process of fibrinolysis and its relation to other physiologic processes involving the mechanism of blood coagulation, protein metabolism and the body's response to various stimuli have been appreciated only recently. The significance of such relationships are discussed in an excellent review of the subject by MacFarlane and Biggs (*Blood* 3: 1167, 1948).

H.W.B.

THE CONCENTRATION OF THE LABILE FACTOR OF THE PROTHROMBIN COMPLEX IN HUMAN DOG AND RABBIT BLOOD, ITS SIGNIFICANCE IN THE DETERMINATION OF PROTHROMBIN ACTIVITY. *A. J. Quick and M. Stefani*. From the Department of Biochemistry, Marquette University School of Medicine Milwaukee, Wisconsin. *J. Lab. & Clin. Med.* 33: 819-826, 1948.

A simple method for assaying the concentration of the labile factor of the prothrombin complex in blood is presented. This method is based on the principle that tricalcium phosphate when added to plasma removes components A and B of the prothrombin complex thus leaving fibrinogen and the labile factor as the only known plasma constituents playing a role in the process of clotting. On adding fresh plasma thus treated to stored human, dog or rabbit blood the prothrombin time was found to shorten strikingly. By determining the amount of plasma that had to be added to a fixed amount of stored plasma in order to reduce the prothrombin to an arbitrarily selected value (20 seconds) the relative concentration of the labile factor could be calculated. By this procedure it was found that the prothrombin time was reduced to a markedly shorter value when the labile factor was added to stored plasma than when added to fresh plasma, thus suggesting that something is elaborated in stored plasma which enhances the activity of the labile factor.

G.E.C.

A COAGULATION DEFECT PRODUCED BY NITROGEN MUSTARD. *T. R. Smith, L. O. Jacobson, C. L. Spurr, J. G. Allen, and M. H. Block*. From the Departments of Medicine and Surgery, the University of Chicago. *Chicago, Illinois Science* 107: 474, 1948.

Five patients were injected with nitrogen mustard (methyl-bis (beta-chloroethyl) amine hydrochloride) as follows: 2 were given 0.1 mg/kg on four successive days, 1 received the same dose and four injections at twelve hour intervals, 1 was given the same dose and four treatments at seven hour intervals and 1 was given 0.3 mg/kg two doses at six hour intervals. Within two weeks all 5 patients developed a moderate anemia, severe leukopenia, thrombocytopenia, prolonged bleeding time, cutaneous petechiae and ecchymoses. Coagulation time was prolonged. Intravenous injections of toluidine blue or protamine brought the coagulation time back to normal. The authors point out that nitrogen mustard treatment may induce serious or fatal complications due to the presence of an anticoagulant in the blood.

R.C.C.

MEASUREMENT OF THE ELECTRIC RESISTANCE OF HUMAN BLOOD. USE IN COAGULATION STUDIES AND CELL VOLUME DETERMINATIONS. *R. L. Rosenthal and C. W. Tobias*. From the Division of Medical Physics and the Department of Chemistry, University of California, Berkeley, California. *J. Lab. & Clin. Med.* 33: 1110-1122, 1948.

A method is described for the measurement of electric resistance of blood and other fluids. Lightly platinumized platinum electrodes were placed in tubes of blood. An audiofrequency oscillator was used to generate power. An oscilloscope was used instead of the conventional telephone bridge balance indicator. By means of a selector switch and parallel circuits six different samples could be studied at one time. All determinations were made in a constant temperature water bath set at 37°C.

Determination of resistance changes during the coagulation of blood make possible the determination of clotting time with elimination of inconsistencies caused by motion and offer a quantitative means for the study of clot retraction. By means of the ratio of blood resistance to plasma resistance the cell volume fraction of a sample of blood may be calculated. It was found that the centrifugation rate was 77 per cent too high (average) a value comparable to that obtained by Chapin and Ross by entirely different techniques (*Am. J. Physiol.* 137: 447, 1942).

G.E.C.

**STUDIES OF HEMOPHILIA I THE CONTROL OF HEMOPHILIA BY REPEATED INFUSIONS OF NORMAL HUMAN PLASMA** *B. Alexander and G. Landwehr* From the Medical Research Laboratories, Beth Israel Hospital and the Department of Medicine, Harvard Medical School, Boston, Mass. *J. A. M. A.* 138: 174-179, 1948

The authors report the prophylactic use of serial infusions of normal human plasma in hemophilia. It was found that the intravenous injection of 100 cc. of plasma into a hemophilic was capable of reducing the coagulation time of the blood to normal levels; the effect, however, began to disappear within a few (eight) hours. When 100 to 190 cc. of plasma was used, the effect was still present in twenty-four hours, was beginning to disappear in thirty-six hours, and was completely gone in three days. The use of larger amounts of plasma—up to 750 cc.—did not cause prolongation of the effect. It was further found that the administration of plasma intramuscularly was of little effect, 30 cc. of plasma having less of a coagulant power than 1 cc. given intravenously.

A schedule was therefore devised in which 100 to 180 cc. of reconstituted, freshly processed, frozen normal human plasma was administered intravenously three times a week to patients with hemophilia. Four patients with long histories of bleeding tendency and increased coagulation time were treated in this manner for from ten to twenty months. It was possible to maintain the coagulation times of these patients at high normal levels (15 to 20 minutes) and there was striking clinical improvement with elimination of serious hemorrhages. The only relapses occurred when, for various reasons, the schedule of plasma infusions was temporarily interrupted. The patients were able to work or go to school, indulge in sports, and even, in one case, undergo a surgical operation (tendon transplantation). There was no refractoriness to the plasma; on the other hand, there was no permanence of effect. The incidence of transfusion reactions was 1.2 per cent, and one patient developed mild serum hepatitis.

The clinical results in these cases are so striking as to endorse the authors' schedule of therapy as a beneficial and practical one at least until fractionation of normal plasma provides a consistently potent product for use in hemophilia.

SE

**INFLUENCE OF SULFONAMIDES ON BLOOD COAGULATION** *M. Kubias* From the City Hospital, Prague. *Čas. lékař. Čes.* 86: 291, 1947

Clinical observations seemed to indicate that the sulfonamides affect the blood coagulation. Therefore experiments were made to test this effect in patients treated for gonorrhea with various sulfa drugs. Blood coagulation, bleeding time, and osmotic resistance of red blood cells were systematically followed. In 10 patients treated in this way, the acceleration of blood coagulability was very marked; it appeared immediately following the first day of treatment and lasted for about six to nine days.

M.N.

**BLOOD PROTHROMBIN LEVEL IN PATIENTS SUFFERING FROM DISSEMINATED SCLEROSIS** *J. Lenf and L. Poldřek* From the Clinic of Nervous Diseases, Charles University, Prague. *Čas. lékař. Čes.* 86: 1569, 1947

Blood prothrombin has been determined in 53 patients suffering from disseminated sclerosis. 34 patients (65 per cent) were a little higher than normal in prothrombin content (over 120 per cent); some of these were very high (150 to 180 per cent). The arithmetical mean value of blood prothrombin was 120.6 per cent.

M.N.

## LEUKEMIA AND MALIGNANT LYMPHOMA

**THE HEMOGRAM IN MALIGNANT LYMPHOMATOSIS (HODGKIN'S DISEASE)** *J. Chester and G. Hemmiller* Medizinische Universitätsklinik, Lausanne (Switzerland). *Praxis* 24: 440-442, 1948

The authors studied the hemogram of 56 cases of Hodgkin's disease. The leukocyte count was subject to important fluctuations and specially in the terminal phase of the disease, leukopenia was more common than leukocytosis.

Lymphopenia was the most frequent symptom; the authors give figures of 60 per cent initially and 90 per cent terminally in the illness. Eosinophilia was less frequent (12 per cent); the same was so for the frequently described monocytosis.

Anemia was seldom seen at the beginning and always developed sooner or later during the course of the disease.

C.M.

**STUDIES IN HODGKIN'S SYNDROME VII NITROGEN MUSTARD THERAPY** R. P. Zares C. A. Dean and H. A. Hoster From the Department of Medicine and the Division of Cancer Research Ohio State University, Columbus, Ohio *J. Lab. & Clin. Med.* 33: 1002-1018, 1948

Thirty-one cases of Hodgkin's disease were treated with a total of 44 courses of methyl bis( $\beta$ -chloroethyl) amine hydrochloride. Beneficial results were observed in 20 patients receiving twenty-four courses. Indirectly, 3 other patients benefited through an apparent resensitization to roentgen rays. Improvement was characterized in most instances by an immediate disappearance of fever, itching and pain. Brownish pigmentation of the skin was observed to decrease in several cases as did Hodgkin's skin lesions, splenomegaly, hepatomegaly and adenopathy. A regeneration of lymphocytes and a return of the monocyte lymphocyte ratio toward normal was the most consistent laboratory finding associated with a clinical remission. Bone marrow hypoplasia proceeding to aplasia and followed in every instance by complete regeneration to the previous level and in some cases to a more normal level within a few weeks after therapy was observed.

G. E. C.

**NITROGEN MUSTARDS IN FOWL LEUCOSIS** E. P. Johnson From the Section of Animal Pathology Virginia Agricultural Experimental Station Blacksburg Virginia *Science* 107: 40-42, 1948

This experiment was performed to determine the effects of nitrogen mustards on the leukosis of fowls. Chicks of 1 to 2 weeks of age were injected with the Belville strain A leukosis virus either intravenously or intraperitoneally. After the leukosis had become established (four to six weeks) chicks were treated with  $\text{HN}_2$  or  $\text{HN}_3$ . Optimal dose of  $\text{HN}_2$  was found to be 1.0 mg/Kg. for  $\text{HN}_2$ , 2.0 mg/Kg. Of 14 birds treated with  $\text{HN}_2$ , 2 made clinical recoveries lasting from three to six months. Of 19 birds treated with  $\text{HN}_3$ , 11 made complete recoveries. With treatment early in the disease the recovery is greater. This work indicates that two nitrogen mustards have a profound action upon the immature cells called hemocytoblasts, retard the mitotic activity both of the blood and the bone marrow, and have a lethal effect upon the virus which causes the disease as indicated by the failure of blood drawn from the treated animals to infect a normal host.

R. C. C.

**PULMONARY EDEMA IN LEUKEMIC MICE FOLLOWING TREATMENT WITH URETHANE** W. W. Winchester and G. M. Higgins From the Division of Experimental Medicine Mayo Foundation Rochester Minnesota *Science* 107: 568-569, 1948

This paper was a study on the effects of urethane on pulmonary capillaries in the mouse. The mice used were F<sub>1</sub>NH hybrids transplanted with myelogenous leukemia. Urethane was administered intraperitoneally after the leukocyte counts were in the neighborhood of 200,000 cells per cu. mm. Doses of urethane varied from 0.5 mg./Gm. daily to 1.0 mg./Gm. daily. Pulmonary edema was present in all treated animals. Giving graded doses produced edema in all cases in which the dose of urethane was sufficient to have an effect on the leukemia. If death did not result from the edema, a subsequent development of pneumonia did produce death. Although the edema was restricted to the lungs, evidence was found of capillary damage in other regions of the body. The authors point out the toxic effects of urethane when used over a long period of time.

R. C. C.

**URETHANE INDUCED LYMPHOPENIA IN NORMAL AND ADRENALECTOMIZED RATS** A. Dwyer and E. D. Rabin From the Department of Physiology George Washington University, Washington D. C. *Endocrinology* 42: 320-325, 1948

Urethane has been used in the treatment of leukemia. This substance has been reported as inducing a lymphopenia as well as other effects. Urethane treatment also induces an adrenal hypertrophy. In view of the work of Dougherty and White where a lymphopenia was induced by adrenal cortical extracts, this work was done to determine whether urethane induces the lymphopenia via the adrenal cortex. Adult male rats of the Sprague Dawley strain were used.

A leukopenia and an absolute lymphopenia were induced by urethane by these authors in both normal and adrenalectomized rats. The adrenals would therefore not seem to be the factor which induces lymphopenia under urethane treatment. The authors discuss the theory that urethane acts as a bone marrow poison.

F. C. C.

THE CHANGE IN THE LEUCOCYTIC FORMULA BY THE LEUCOCYTOSIS-PROMOTING FACTOR OF EXUDATES IN EXPERIMENTAL LEUCEMIA *V Menkin* From the Agnes Barr Foundation for Cancer Research Temple University School of Medicine, Philadelphia, Pennsylvania *Science* 107 546-547 1948

Leukemia was induced in mice by injections of leukemic material. A few days to a few weeks later the mice were injected subcutaneously with 1.2 mg. of leukocytosis-promoting factor (obtained from canine exudates as previously described by the author). Injections were daily at first and, after several weeks, three times per week. This material induced a shift in the differential leukocyte formula with a rise in the percentage of mature polymorphonuclear leukocytes. Several children with leukemia were injected with this material. The only positive effect was a frequent drop in the total leukocyte level. R.C.C.

REGRESSION OF LYMPHOSARCOMA PRODUCED BY INTRAPERITONEAL ADMINISTRATION OF 95% ETHYL ALCOHOL. *A D Bass and M L H Freeman* From the department of Pharmacology Syracuse University College of Medicine Syracuse New York. *Science* 107 114-115, 1948

This experiment was performed in an attempt to determine whether the effects of alcohol on lymphosarcoma were due to a direct effect or to effects mediated by the adrenal cortex. C<sub>3</sub>H mice bearing 6C<sub>3</sub>HED tumors were used. One group of mice was treated with 19 per cent ethyl alcohol and another group was treated with various amounts of 95 per cent alcohol. The 19 per cent alcohol produced no toxic symptoms and no tumor regression, while the 95 per cent alcohol did produce toxic symptoms and showed a definite tumor regression. Diffuse cell necrosis was seen in the tumors treated with the 95 per cent alcohol. The results did not answer the original question as to whether the adrenal cortex was involved. The authors suggest that the results obtained were not due to the alcohol itself but were due to the toxic effects obtained. R.C.C.

SYSTEMIC ALEUKEMIC RETICULOENDOTHELIOSIS (LETTERER-SIWE DISEASE) *C Varga, M N Rubter and A G DeSanctis* From the Department of Pediatrics and the Department of Pathology New York Post Graduate Medical School and Hospital New York. *Am J Dis Child* 75 376-384 1948

Three cases of systemic aleukemic nonlipid reticuloendotheliosis are reported with a brief discussion of the disease. The usually accepted criteria of this disease are (1) its occurrence, neither hereditary nor familial, in infants and young children, of unknown etiology and fatal prognosis, (2) hepatosplenomegaly, (3) generalized lymphadenopathy, (4) hemorrhagic diathesis, (5) localized skeletal changes or tumors, (6) progressive secondary anemia, usually with a normal leukocyte count, (7) general hyperplasia of the cells of the reticulo-endothelial system which may assume focal tumor-like proliferation, and (8) an acute onset unrelated to infections. The authors take issue with this last criterion as their cases, like many others reported in the literature, were associated with, although not necessarily caused by, infection. Emphasis was also placed on the presence of cutaneous lesions similar to seborrheic dermatitis which so frequently occur in the systemic reticulo-endothelial diseases.

Certain authors, including Siwe, have made a distinction between this disease and so-called infectious reticuloendotheliosis, which in all other respects appear to be similar. The very frequency of infections in young children as well as those occurring coincidentally in malignant disease make such a distinction extremely tenuous. One wonders about the precise relationship of this disease to Schüller-Christian disease, leukemic reticuloendotheliosis, reticulum cell sarcoma, etc., and whether it should be rightly considered a separate disease entity. Certainly our whole concept of the reticulo-endothelial disorders is confusing and badly in need of clarification. H W B

LYMPHOCYTE DISCHARGE FROM THE ISOLATED RABBIT SPLEEN BY ADRENAL CORTICAL EXTRACT *O Habtin* From the Worcester Foundation for Experimental Biology Shrewsbury, Mass. and the Department of Physiology Tufts Medical College Boston, Mass. *Endocrinology* 42 285-306, 1948

The purpose of this experiment was to determine the effect of adrenal cortical extract (ACE) on the lymphocyte content of the spleen under *in vitro* conditions. Rabbits served as the source of the spleens and the blood. A perfusion apparatus was used, the details of which are given. ACE administered to the isolated rabbit spleen under conditions of constant pressure and bathed by whole blood produced a

significant rise in the lymphocyte content of the blood. This rise occurred rapidly (15 minutes). The lymphocytes then dropped below normal levels. This secondary decrease in the circulating lymphocytes appears to be due to accelerated lymphocyte breakdown by the spleen, in the presence of ACE. These reactions to ACE were not dependent on changes in pressure or splenic blood flow. They could not be elicited with the thymus, lung or liver. Glucose, epinephrine, desoxycorticosterone acetate and estradiol propionate had no effect on the circulating lymphocytes.

R.C.C.

**SOLENIC LYMPHOCYTE DISCHARGE INDUCED BY ADRENAL CORTICAL HORMONES UNDER IN VIVO CONDITIONS**  
D. Stone and O. Hechter. From the Worcester Foundation for Experimental Biology, Shrewsbury, Mass., and the Department of Physiology, Tufts Medical School, Boston, Mass. *Endocrinology* 42: 307-314, 1948.

In a previous paper it was noted that adrenal cortical extract (ACE) produced a discharge of lymphocytes into the circulation from the spleen under *in vitro* conditions. This work was done to study the spleen under *in vivo* conditions. Rats were used for the experiment and a stress was obtained by making the rats swim. A lymphocytosis resulted from this stress in normal animals. This rise was significantly decreased by adrenalectomy or splenectomy. Injections of ACE to adrenalectomized swimming rats produced a lymphocytosis. ACE injections had no effect on adrenalectomized splenectomized swimming rats. Desoxycorticosterone acetate had no effect. ACE, then, would seem to induce lymphocyte discharge from the spleen under *in vivo* conditions as well as under *in vitro* conditions. This work is interesting when compared to the lymphopenia obtained by ACE in previous reports by Dougherty and White. Is the breakdown of the lymphocyte in lymph nodes greater than the discharge from the spleen when animals are injected with ACE?

R.C.C.

**LYMPHOPENIA FOLLOWING ELECTRICALLY INDUCED CONVULSIONS IN MALE PSYCHOTIC PATIENTS. IV**  
P. Mikkelsen and T. T. Hutchins. From the United States Veteran Administration Hospital, American Lake, Washington. *Endocrinology* 42: 394-398, 1948.

The object of this experiment was to determine if the stress of an electrically induced convulsion would produce a lymphopenia in psychotic patients. A significant lymphopenia was found in the third hour following both the grand mal and petit mal reactions. The relation of these results to the lymphopenia found after other stresses and after injections of adrenal cortical extract or adrenocorticotrophic hormone are discussed.

R.C.C.

## IMMUNOHEMATOLOGY

**PRELIMINARY NOTE ON INFLUENCE OF HETERO SPECIFIC IMMUNIZATION ON PRODUCTION OF RH ANTIBODIES**  
J. J. van Loghem. Centraal Laboratorium van den Bloedtransfusie dienst, Binnengasthuis, Amsterdam. *Brit. M. J.*, 2: 316-318, 1948.

Volunteers were given repeated intravenous injections of red cells in an attempt to induce anti Rh agglutinins. When, after 15 to 41 injections, no antibodies were found mixed typhoid, paratyphoid vaccines were given simultaneously.

The relatively weak antigens C and E were used. Rh antibodies appeared in 4 of 17 volunteers, 2 of these only after vaccine injections. One of the others showed a striking increase in titer after the vaccine. In general, only those who showed a clinical response to the vaccines and who produced antibodies to nearly all the injected typhoid antigens showed a satisfactory Rh antibody response.

If this observation can be confirmed on a larger scale the technique should prove most useful both for producing anti sera and as the author suggests, for screening for potentially good antibody producers.

S.T.C.

**GENETIC TRANSMISSION OF TWO RARE BLOOD GROUP GENES**  
A. S. Wiener. Jewish Hospital, Brooklyn, N. Y. *Nature*, London 162: 735, 1948.

This note records the phenotypes and genotypes of four families, three of whom show transmission of the gene R<sup>s</sup> and one the extremely rare r<sup>s</sup>.

S.T.C.



**INTRAGROUP INCOMPATIBILITY WITH RESPECT TO THE H<sub>r</sub> BLOOD FACTORS AS A CAUSE OF MINOR HEMOLYTIC TRANSFUSION REACTIONS** *A S Wiener* From the Blood Transfusion Division of the Jewish Hospital of Brooklyn and the Serological Laboratory of the Office of the Chief Medical Examiner New York New York *J Lab & Clin Med* 33 985-997 1948

At the author's institution, the frequency of post transfusion febrile reactions has been reduced from 7.9 per cent in 1936 to only 1.2 per cent in 1947 as a result of the perfection of methods of eliminating pyrogenic materials from blood transfusion apparatus. This virtual elimination of pyrogenic reactions has served to make more prominent mild hemolytic reactions occurring in Rh positive patients as a result of H<sub>r</sub> sensitization by repeated transfusions given over a long period of time. In a series of 23 Rh positive patients having febrile reactions and at the same time showing evidence of posttransfusion hemolysis 17 were H<sub>r</sub> negative. Among 10 patients with febrile reactions but without evidence of hemolysis one was H<sub>r</sub> negative. The author suggests that one should investigate every febrile reaction for evidence of hemolysis. If hemolysis has occurred even though the patient is Rh positive, H<sub>r</sub> tests should be done and if the patient is found to be H<sub>r</sub> negative, only H<sub>r</sub>-negative blood of a compatible blood group should be used for future transfusions. If Rh negative patients have reactions despite transfusions of type rh blood, one should search for other sensitizations, particularly against the M factor.

G.E.C.

**SPECIFIC SERUM AGGLOUTINATION OF ERYTHROCYTES SENSITIZED WITH EXTRACTS OF TUBERCLE BACILLI** *G Middlebrook and R J Dubos* From the Laboratories of the Rockefeller Institute for Medical Research New York, New York *J Exper Med* 88 521-528, 1948

Sheep's erythrocytes sensitized with extracts of human tubercle bacilli or products of their culture filtrate were agglutinated by sera of rabbits previously injected with BCG and by sera of patients with active pulmonary tuberculosis. At least one material capable of sensitizing the red cells was shown to be heat stable and present in the polysaccharide fraction of the tubercle bacillus. Evidence for the specificity of this hemagglutination was obtained from the negative or insignificant reactions observed when the sensitized red cells were tested against sera of experimental animals immunized with other bacteria and against sera of nontuberculous individuals. It was of particular interest that there was no cross-reaction with Wassermann positive sera.

Inhibition of the specific hemagglutination reaction was accomplished by adding the soluble reactive antigen to the serum before the red cells were introduced into the system. Utilization of both the inhibition test and the agglutination test permitted the detection and quantitation of small amounts of the sensitizing antigen.

The authors have suggested the possibility that this method may be of aid in the detection of a specific antigen circulating in vivo and that there may be even some correlation between the degree of activity of tuberculosis and the titer of the patient's serum in the hemagglutination test.

H.W.B.

## ERRATA

In Wiener, Alexander S., and Wexler, Irving B. Results of therapy of erythroblastosis with exchange transfusions. *Blood* 4: 1-35 (January), 1949.

Page 8, second line from bottom, 'the Rh<sub>0</sub> factor' (instead of "the Rh<sub>1</sub> factor")

Page 12, third line from bottom "A<sub>1</sub>MRh<sub>1</sub>rh" (instead of "A<sub>1</sub>MRh<sub>1</sub>h")

Page 35, first word of second line of reference 24 potent (instead of "parent")

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Assistant Professor of Medicine, Harvard Medical School, Director of Internal Medicine and Research in Clinical Physiology McLean Hospital Waverly Mass., Visiting Physician and Associate in Research, Beth Israel Hospital Boston, Mass.

HELEN ASHENBRUCKER, B.A.

Department of Medicine School of Medicine University of Utah, Salt Lake City, Utah

M. BESSIS, M.D.

Director of the Research Laboratory of the National Center of Blood Transfusion, Paris France

MARY C. BUCCIERO, M.S.

Biochemist Harper Hospital, Detroit, Mich.

G. E. CARTWRIGHT, M.D.

Department of Medicine, School of Medicine, University of Utah, Salt Lake City Utah

WILLIAM DAMESHEK, M.D.

Hematologist, J. H. Pratt Diagnostic Hospital Professor of Clinical Medicine Tufts College Medical School, Boston, Mass.

JAMES M. ORTEN, Ph.D.

Associate Professor Department of Physiological Chemistry, Wayne University College of Medicine, Detroit, Mich.

ROBERT B. PENNELL, Ph.D.

Department of Immunochemistry Division of Medical Research Sharp & Dohme Inc., Glenolden Pa.

WILLIAM ELLIOTT SMITH, B.S.

Department of Immunochemistry Division of Medical Research Sharp & Dohme Inc., Glenolden Pa.

TOBIAS STEIN, M.D.

Chief of the Medical Service Veterans Administration Hospital, Montgomery, Ala.

BETTY TATTING, B.A.

Department of Medicine School of Medicine University of Utah, Salt Lake City Utah

BERT L. VALLEE, M.D.

Research Associate, Department of Biology Massachusetts Institute of Technology, Cambridge Mass., Research Fellow in Medicine Harvard Medical School, Boston, Mass.

LOUIS WEISFUSE, M.D.

Assistant Department of Medicine Long Island College of Medicine, Brooklyn N.Y.

M. M. WINTROBE, M.D., Ph.D.

Professor and Head, Department of Medicine School of Medicine University of Utah Salt Lake City Utah

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# BLOOD

## *The Journal of Hematology*

VOL IV, NO 4

APRIL, 1949

### EXPERIMENTAL PRODUCTION OF A NUTRITIONAL MACROCYTIC ANEMIA IN SWINE

By G E CARTWRIGHT, M D, BETTY TATTING, B S, HELEN ASHENBRUCKER, B A,  
AND M M WINTROBE, M D, Ph D

THE PURPOSE of this paper is to describe the experimental production of a macrocytic anemia in swine made deficient in pteroylglutamic acid. This subject is of interest because of the morphologic resemblance of the experimental anemia to pernicious and related macrocytic anemias, and because it is known that the hematologic manifestations of these anemias respond to pteroylglutamic acid. A preliminary report of this work has appeared.<sup>1</sup>

#### REVIEW OF THE LITERATURE

Since the contributions of Minot and Murphy in 1927<sup>2</sup> and of Castle in 1929<sup>3</sup> concerning the etiology of pernicious anemia, many attempts have been made to produce macrocytic anemia in animals by a variety of different approaches. However, the entire pernicious anemia syndrome has not yet been produced in the experimental animal.

Several investigators have reported the production of macrocytic anemia in swine. Miller and Rhoads<sup>4</sup> as early as 1935 fed swine a canine black-tongue producing diet and observed a symptom complex which included anemia, lesions of the oral mucous membranes, gastric achlorhydria, diarrhea and motor weakness of the extremities. The disorder was thought to be associated with a loss of the anti-pernicious anemia activity of the gastric secretion and liver. Remissions of the anemia and amelioration of symptoms were induced by the administration of liver extract. Contrary to their claim, however, the anemia was not actually macrocytic. The average mean corpuscular volume in the animals with macrocytic anemia was reported to be 59.5 c. The mean corpuscular volume in normal swine is about  $56 \pm 5.16$  cu.<sup>1</sup> Furthermore, intermittent periods of achlorhydria may be observed in normal swine.<sup>5</sup>

Smith, Reiser and Harrell<sup>6</sup> observed a macrocytic anemia in weanling pigs on a prolonged partial deficiency of the vitamin B complex but spontaneous cure of the anemia ensued while the pigs remained on the same diet and without treatment. The diet used was a modification of the Goldberger black-tongue producing diet.

From the Department of Medicine, College of Medicine, University of Utah, Salt Lake City, Utah.  
Aided by a grant from the United States Public Health Service and the Upjohn Company, Kalamazoo, Michigan.

and was almost identical to that used by Miller and Rhoads.<sup>4</sup> The mean corpuscular volumes reached 74 to 97  $\mu$ . The inclusion of 10 per cent brewer's yeast in the diet protected the animals. McGowan and Sinclair<sup>7</sup> found that young pigs kept on a ration of corn, fish meal and "draff" became ill with anemia and jaundice and liver damage were observed. The anemia was described as macrocytic and the femoral marrow was red and cellular. Reticulocyte increases occurred following the administration of raw liver, the anemia disappeared and the bone marrow became normal. Lawrason and Cronkite<sup>8</sup> observed a macrocytic anemia and achlorhydria in two pigs exposed to atom bomb ionizing radiation at Bikini. In one animal the bone marrow was hyperplastic and megaloblastic-like. The relation of these instances of anemia in experimental animals to nutritional deficiency is not clear.

Welch, Heinle and colleagues<sup>9-11</sup> have reported the production of macrocytic anemia with megaloblastic hyperplasia of the bone marrow in pigs maintained on a highly purified diet essentially free of extrinsic factor and to which sulfasuxidine and a folic acid antagonist were added. One of the animals responded to the administration of crude sodium caseinate together with a 95 per cent ethanol extract of crude casein and normal human gastric juice. This animal later relapsed and was treated successfully with a single injection of 15 units of liver extract. A second animal responded rapidly to four daily intramuscular injections of 10 mg. of pteroyl glutamic acid. A third pig became critically ill and was successfully treated with a combination of purified liver extract, pteroylglutamic acid and niacinamide. These investigators concluded that purified liver extract is effective in correcting the anemia initially but found that relapses developing after liver-induced remissions were refractory to liver extract.<sup>11</sup> Extract prepared from the liver of a pig raised on the purified casein diet and given pteroylglutamic acid was hemopoietically inactive when assayed in a patient with pernicious anemia. An extract from the liver of the same animal after a month on the same diet, except that crude casein containing extrinsic factor replaced the purified casein, was active when so assayed. An extract prepared from normal pig liver was even more active. It was concluded that pteroylglutamic acid can elicit a complete hematopoietic response in pigs on a purified diet poor in extrinsic factor.<sup>11</sup> Our own studies in swine<sup>1</sup> have been alluded to already and will be discussed more fully later.

In monkeys, Wills reported<sup>12-15</sup> the production of a severe macrocytic anemia with a megaloblastic bone marrow. The animals were given a diet similar to that in common use among the poorer class of Mohammedans in Bombay, where nutritional macrocytic anemia is common. It consisted of polished rice, margarine, salt, iron, white bread, cod liver oil, and either tomatoes or carrots. The anemia responded rapidly following the administration of either marmite or Campolon but purified anti-pernicious liver extracts were ineffective. Bartonella infections were not present.

The earlier work in dogs fed a modified Goldberger black-tongue producing diet was inconclusive.<sup>16</sup> Several of the dogs had been splenectomized and it was later demonstrated that the anemia was complicated by Bartonella infection and that such an infection was capable of itself producing a severe anemia.<sup>17</sup> Later a rela-

relationship between nicotinic acid deficiency and the development of macrocytic anemia was suggested by the work of Handler and Featherston.<sup>18</sup> Dogs fed three different types of diet deficient in nicotinic acid developed severe macrocytic anemia. A sharp reticulocyte response and subsequent elevation of the red cell count and hemoglobin followed the administration of nicotinic acid but not when purified liver extract was given. More recently Ruegamer, Brickson, Torbet and Elvehjem<sup>19</sup> observed that when young growing dogs were fed a niacin-deficient purified ration containing 1 per cent sulfasuxidine, weight loss and signs of black-tongue developed. Small doses of niacin were only partially effective in combating the loss of weight. Pteroylglutamic acid helped to produce a more consistent response to niacin but had no apparent effect on the macrocytic anemia which appeared and became progressively more severe. Diarrhea was present and in the most deficient animals a pronounced, flaccid type of paralysis was observed. As little as 0.05 ml. of purified liver extract (20 units/ml) was effective in bringing about a complete remission of the anemia. Bone marrow studies were not mentioned. This work would seem to be of considerable importance if it can be confirmed since it represents the only liver extract responsive, macrocytic anemia associated with neurological symptoms to be reported to date in experimental animals. The administration of choline<sup>20</sup> or acetylcholine<sup>21</sup> has been claimed by Davis to produce a macrocytic anemia in dogs but this has not been confirmed.<sup>22</sup>

Wills<sup>12</sup> produced macrocytic anemia in rats but found that the anemia was due to Bartonella infection. Watson, Cameron and Wits<sup>23</sup> have reported that the formation of a blind intestinal loop in rats leads to the development of a macrocytic anemia which responds to purified liver extract. The bone marrow contained increased numbers of proerythroblasts and basophilic erythroblasts. A striking difference between this anemia and that seen in nutritional macrocytic anemia and in pernicious anemia is the fact that marked reticulocytosis (10 to 40 per cent) was present in the rats prior to therapy.

It has been reported that in some instances a deficiency of copper in sheep<sup>24</sup> and cattle<sup>25</sup> is associated with a macrocytic anemia but detailed morphologic studies have not been done and this work needs confirmation.

Pteroylglutamic acid deficiency in the rat<sup>6, 23</sup> is associated with severe normocytic anemia, granulocytopenia, lymphopenia and thrombocytopenia. However, such a deficiency in the chick has been described as leading to the development of macrocytic anemia<sup>26</sup> and the mean corpuscular volume has been reported to be increased from the normal of 137  $\mu$  to 161  $\mu$ . The anemia is accompanied by severe leukopenia, due mainly to lymphopenia, the absolute numbers of neutrophils being maintained. The thrombocytes also diminish in number. In none of these species has the anemia responded to purified liver extract therapy.<sup>6, 23</sup>

The entire stomach of various species of animals including the dog, cat, rat, pig and monkey has been removed by a number of investigators. This approach has not resulted in the production of macrocytic anemia in any species.<sup>27</sup> Petri and his group<sup>28</sup> in a large series of experiments extending over a period of nearly twenty years have failed to produce macrocytic anemia in either the dog or the pig. They

have observed, however, that total gastrectomy in swine leads to the complete disappearance of the anti-pernicious anemia substance in the liver and that nicotinic acid therapy subsequent to the resection can prevent this loss<sup>23</sup>

### EXPERIMENTAL PROCEDURE

Including the four animals described in the preliminary report,<sup>1</sup> a total of 32 weanling Chester White pigs 21 to 28 days of age were used in this study. The animals were housed in individual cages and were fed the purified diet from the day they were received which was also the day of weaning.

Two types of basal diet were fed, a 10 per cent and a 26 per cent protein diet the compositions of which were as follows

	per cent	per cent
Casein	26.1	10.0
Sucrose	57.7	73.8
Lard	11.0	11.0
Salt mixture <sup>24</sup>	5.2	5.2

Two types of casein were used in different animals. Sheffield's New Process (crude) casein and Sheffield's alcohol-extracted (purified) casein. The latter was prepared from Sheffield's high nitrogen casein<sup>25</sup> by presoaking for 18 hours with cold 98 per cent methanol at pH 6 followed by a continuous 24 hour extraction with hot methanol. The extracted casein was then tray dried to remove the residual methanol.

It has been demonstrated previously in this laboratory by assay in patients with pernicious anemia to relapse that the Sheffield New Process (crude) casein contains significant amounts of extrinsic factor activity.<sup>1</sup> The Sheffield alcohol-extracted (purified) casein has been assayed in a similar manner for extrinsic factor activity. The procedure of assay was as follows. The patient was hospitalized and during the assay periods liver, meat, meat products, milk and poultry were excluded from the diet. Bread, cereals, sugar, fats, vegetables and fruits were permitted in the amounts desired. Daily for ten days 50 grams of the casein to be assayed were incubated at 37°C for two hours with 150 to 200 ml of normal human gastric juice at pH 2.5 to 3.5. The incubation mixture was then strained through cheese cloth, the filtrate was neutralized to pH 5.0 and administered immediately to the patient. The results are shown in figure 1. The purified casein in the quantity given contained insignificant amounts of extrinsic factor activity whereas the same quantity of crude casein apparently carried an amount adequate to give a significant reticulocytosis and rise in volume of packed red cells. Following this response the administration of one U.S.P. unit of purified liver extract daily for ten days did not produce a further reticulocytosis.

Depending upon the type and amount of casein in the basal diet the animals were divided into four groups as follows:

Group A—Crude casein 10 per cent

Group B—Crude casein 26 per cent

Group C—Purified casein 10 per cent

Group D—Purified casein 10 per cent plus 15 U.S.P. units of purified liver extract (61039 Parke Davis 15 U.S.P. units per ml) administered intramuscularly every 15 days from the beginning of the experiment.

The basal diet was fed in amounts of 365 grams (152 calories) per kilogram of body weight per pig per day. Sulfasuxidine was added to the basal diet of all animals in amounts of 2.0 per cent. All animals were given a crude methyl folic acid antagonist prepared† by allowing 2.45 triamino-6-hydroxyprimidine and p-amino benzoyl L (+)-glutamic acid to react with 2,3-dibromobutyraldehyde.<sup>26, 27</sup> This antagonist was administered daily either in capsules (0.06 Gm per kilogram of body weight) or added to the diet (0.2 per cent). Natola (Parke, Davis 5500 units of vitamin A, 11000 units of vitamin D per gram) 0.056 gram per kilogram of body weight per week supplemented the basal diet. Vitamins

\* Prepared by heating freshly separated fat free milk to 110–115°F and precipitating the casein with dilute muriatic acid at pH 4.5–4.6. The casein is then repeatedly washed to reduce the free acid and mineral constituents to a minimum and is then dried continuously.

† By Dr. M. E. Holtquist and Dr. J. M. Smith, Jr. of the Calco Chemical Division, American Cyanamid Company, Pearl River, N. Y.

were supplied in crystalline form by placing them in capsules and administering them orally three times a week. The quantities given were as follows (mg per kilogram of body weight per day)

Thiamin hydrochloride	0.25	Pyridoxine hydrochloride	0.20
Riboflavin	0.12	Calcium pantothenate	0.50
Nicotinic acid	1.20	Choline chloride	10.00

In addition all animals received crystalline biotin 50  $\mu$ g per kilogram of body weight per week intramuscularly.

Hematologic studies (red blood cell count, hemoglobin, volume of packed red cells, red cell indices, reticulocyte count, total leukocyte count, differential leukocyte count and platelet count) were performed weekly on each animal throughout the entire experiment.

The cellular composition of the bone marrow was studied in each animal at the onset of the experiment and at the time of development of the deficiency, as well as before and after each therapeutic test. Specimens of bone marrow were obtained by aspiration of the sternal marrow with standard 16 gauge sternal puncture needles. A small amount of marrow fluid, usually less than 0.3 ml, was withdrawn into a clean dry syringe and then cover glass preparations were drawn and stained with Wright's stain. Differential cell counts were made on 500 to 1,500 cells. Because of the large amount of material the differential counts

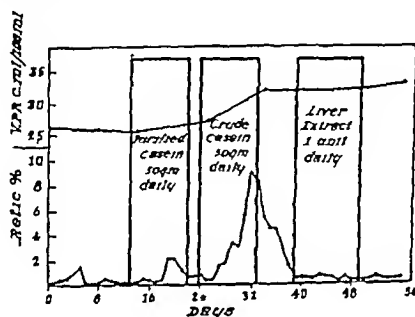


FIG. 1.—Assay of purified and crude casein for extrinsic factor activity in a patient with pernicious anemia in relapse. The purified casein contained minimal extrinsic factor activity whereas 50 grams of the crude casein contained an amount presumably equivalent to 1 USP unit of liver extract, since no additional response followed the administration of this amount of liver extract.

will not be presented in detail in this publication. Photomicrographs of several types of cells as well as detailed differential counts were presented in the preliminary report.<sup>1</sup>

The total 24 hour urinary excretion of total hydroxyphenyl compounds (tyrosine, p-hydroxyphenyl lactic and p-hydroxyphenylpyruvic acids) expressed as the tyrosine equivalent and referred to as "tyrosyl" was determined by the Folin and Ciocalteu method<sup>24</sup> as modified by Medes.<sup>25</sup> The urine made weakly acid with acetic acid was shaken with fuller's earth to remove coloring matter and impurities capable of reacting with mercuric sulfate in subsequent procedures.<sup>26</sup> The intensity of the final color was measured in the Evelyn photoelectric colorimeter using filter 520 m $\mu$ . Commercial tyrosine, three times recrystallized,<sup>26</sup> served as a standard. Allantoin was determined in the urine by the method of Young and Conway,<sup>27</sup> using the Evelyn photoelectric colorimeter and filter 520 m $\mu$ . Recrystallized potassium allantoinate served as a standard. Urinary uric acid was determined by a modification<sup>28</sup> of the Kern and S. analysis method.<sup>21</sup>

## RESULTS

**General.** The animals deficient in pteroylglutamic acid presented an untidy appearance with thin, lusterless hair. Their growth was poor, although it was no more impaired than that of the low protein controls not given the antagonist.<sup>1</sup> On the 26 per cent casein diet growth was good but, by comparison with the controls,



TABLE 1.—Summary of the Data on the Anemia

Group	Number of Pigs	Days on Experiment	Prior to Deficiency						During Deficiency							
			R.B.C Mill/ c.mm.	Hgb Gm. %	VRPC ml/100 ml	MCV cμ	MCH γγ	MCHC %	Redes %	R.B.C Mill/ c.mm.	Hgb Gm. %	VRPC ml/100 ml	MCV cμ	MCH γγ	MCHC %	Redes %
A	12	120 ±26 9	7 76 ± 61	13 2 ± 67	42 1 ±1 77	55 ±3 44	17 ±1 26	31 ± 77	1 0 ± 72	3 56 ± 63	8 1 ±1 50	25 6 ±4 33	73 ±3 41	23 ±1 55	32 ±1 10	2 7 ±1 18
B	4	160 ±37 2	7 12 ± 68	11 9 ± 26	38 3 ± 90	54 ±5 35	17 ±1 53	31 ± 57	1 1 ± 12	3 28 ± 49	9 2 ± 89	27 1 ±2 10	84 ±8 40	28 ±3 22	34 ±1 15	1 7 ±1 20
C	11	69 ±11 4	8 19 ± 80	13 4 ±1 37	41 8 ±2 75	51 ±3 45	16 ±1 48	32 ±1 79	0 7 ± 53	3 89 ±1 16	7 6 ±2 29	23 2 ±6 95	60 ±2 94	19 ±1 85	33 ±1 98	2 5 ± 62
D	5	76 ±5 8	7 79 ± 68	14 0 ±1 01	42 5 ±1 56	54 ±3 54	18 ±1 00	33 ±1 58	0 5 ± 23	3 55 ± 82	7 6 ±1 70	22 8 ±4 96	65 ±2 29	21 ±1 00	34 ±1 23	2 2 ± 81

Group A Crude casein, 10 per cent Group B Crude casein, 26 per cent Group C, Purified casein, 10 per cent, Group D, Purified casein 10 per cent plus liver extract (15 units every 15 days)

VRPC volume of packed red cells.

MCV, mean corpuscular volume

MCH mean corpuscular hemoglobin

MCHC mean corpuscular hemoglobin concentration

curves of animals fed the basal diet plus 3 to 6 grams of yeast per kilogram of body weight,<sup>4</sup> there was a significant impairment of growth. At 120 days of age the four deficient animals given a 26 per cent casein diet (group B) weighed on the average 24.1 kilograms and at 180 days 46.1 kilograms. The weight of animals given yeast and no antagonist was 25 kilograms at 120 days of age and 67 kilograms at 180 days.

In addition, the deficient animals became listless, weak and ate poorly. Moderately severe diarrhea was present and the stools were somewhat orange-yellow in color, due presumably to the presence of antagonist. No oral lesions were observed. Spontaneous partial remissions of the pancytopenia occurred from time to time in the course of the experiment (figures 6 and 7). These remissions were usually associated with a slight reticulocytosis of 6 to 8 per cent. It is unlikely that the remissions were due to contamination of the diet since only one of two pigs kept in separate pens but eating out of a common trough might have a spontaneous response. A more plausible explanation would be that a favorable change in the synthesis of pteroylglutamic acid by the intestinal flora took place at times. Once the deficiency had become fully established, however, no further spontaneous remissions were observed.

It should be noted that the four pigs (10-53, 10-54, 10-56 and 10-64) described in the preliminary report<sup>1</sup> were depleted of pteroylglutamic acid for 80 to 120 days before the crude methyl folic acid antagonist was administered. These four pigs are included in group A. The remainder of the animals described here were given the antagonist from the beginning of the experiment.

**Red Blood Cells.** All four groups of animals developed severe macrocytic anemia (table 1), the only difference between the groups being in the rapidity of development and degree of macrocytosis. The administration of 26 per cent crude casein (group B) rather than 10 per cent crude casein (group A) delayed the onset of anemia by an average of 40 days. Anemia developed most rapidly in the animals receiving 10 per cent purified casein (group C), severe anemia being present in about 69 days as compared with the 160 days required in the animals fed 26 per cent crude casein (group B). As can be seen by inspection of the values for group D (table 1) the administration from the beginning of the experiment of 15 U.S.P. units of liver extract every 15 days did not prevent nor delay the appearance of anemia. The animals in group D were given a diet identical with that of group C.

The anemia in all four groups of animals was macrocytic. The mean corpuscular hemoglobin concentration was normal. However, the macrocytosis in group B was marked, whereas in group C it was only slight. The factors which seemed to determine the degree of macrocytosis are illustrated in figure 2. From this and from inspection of table 1 it can be seen that the degree of macrocytosis increased as time went on. An additional factor appeared to be the amount of protein in the diet since the group receiving 26 per cent casein (group B) developed a greater degree of macrocytosis in the same period of time than did the group receiving 10 per cent casein (group A). In the former group mean corpuscular volumes as great as 104  $\mu$  were observed.

The first change noted in the red cells was a marked anisocytosis. At the usual

number of macrocytes and microcytes were present. As the deficiency progressed the proportion of macrocytes increased. Poikilocytosis was not prominent at any time. An increase above the normal in the number of Howell-Jolly bodies, nucleated red blood cells and polychromatophilic cells generally took place. The anemia was associated with a slight reticulocytosis of 2 to 3 per cent (table 1).

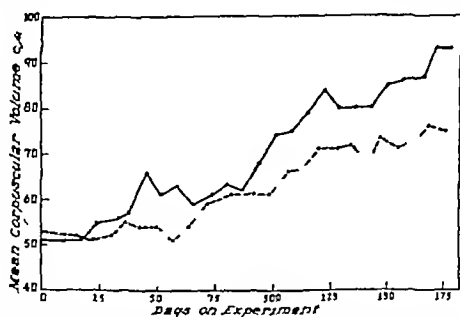


FIG. 2.—Degree of macrocytosis in animals fed the crude, 26 per cent casein diet (Group B, solid line) compared with that in animals fed a crude 10 per cent casein diet (Group A, broken line). The data represent the means of values in all 4 pigs of Group B and 6 pigs studied for a corresponding length of time in Group A.

TABLE 2.—Summary of the Data on Leukocytes and Platelets

Group	Number of Pigs	Days on Experiment	Prior to Deficiency				During Deficiency			
			WBC × 1000 per c.mm.	PMN × 1000 per c.mm.	MNC × 1000 per c.mm.	Platelets × 1000 per c.mm.	WBC × 1000 per c.mm.	PMN × 1000 per c.mm.	MNC × 1000 per c.mm.	Platelets × 1000 per c.mm.
A	12	120 ±26.9	17.5 ±2.43	5.6 ±.70	11.9 ±1.97	573 ±130	7.9 ±1.40	1.4 ±.41	6.5 ±1.19	388 ±294
B	4	160 ±37.2	17.0 ±4.77	5.8 ±.97	11.2 ±4.96	430 ±76	5.6 ±2.91	0.9 ±.91	4.7 ±2.82	240 ±110
C	11	69 ±22.4	17.0 ±2.80	6.3 ±1.72	10.7 ±2.12	548 ±66	8.5 ±3.81	1.0 ±.87	7.5 ±3.24	278 ±214
D	5	76 ±5.8	17.1 ±3.39	6.7 ±2.44	10.4 ±2.58	474 ±111	7.1 ±2.07	1.0 ±.79	6.1 ±1.60	260 ±174

Group A, Crude casein, 10 per cent; Group B, Crude casein, 26 per cent; Group C, Purified casein, 10 per cent; Group D, Purified casein, 10 per cent plus liver extract, 15 units every 15 days.

PMN, polymorphonuclear cells including neutrophils, eosinophils and basophils.

MNC, mononuclear cells including lymphocytes and monocytes.

*Leukocytes* (table 2). Marked leukopenia accompanied the anemia, the absolute number of leukocytes being reduced to 50 to 58 per cent of their original values. The leukopenia resulted from a reduction in both polymorphonuclear and mononuclear cells but there was a proportionately greater reduction in polymorpho-

nuclear cells (52 per cent) than in mononuclear cells (44 per cent). All four groups of animals developed these changes to the same degree but the changes occurred much more rapidly on the purified than on the crude casein. Giant metamyelocytes and multinucleated neutrophils such as those seen in the blood smears of patients with pernicious anemia were not observed in the blood of the pigs.

*Platelets* (table 2). Slight thrombocytopenia developed. The values for individual pigs varied considerably but in general there was a slight reduction at the time of maximal anemia. No differences were noted between the four groups. Spontaneous rises occurred occasionally (figures 6 and 7).

*Sternal Marrow*. Differential cell counts on marrow obtained from the deficient animals revealed rather striking alterations from the normal. These are only summarized in table 3 since detailed differential counts were presented in the preliminary report.<sup>1</sup> No significant differences were noted between the four groups of animals. For this reason all the deficient animals are included in one group in the table.

TABLE 3.—*Summary of Bone Marrow Studies in Eight Pigs<sup>1</sup> Receiving a Complete Diet Low Only in Protein and in Twenty Pyruvylglutamic Acid Deficient Pigs*

Cell Type	Non Deficient Pigs	Deficient Pigs
Myeloblasts	0.9	1.7
Promyelocytes and Myelocytes	9.2	12.1
Metamyelocytes and PMN Neutrophils	49.3	22.2
Normoblasts	32.3	35.2
Megaloblasts	0.0	15.4
Leukocyte-Erythroid Ratio	2.1	1.0

Values represent means

There was a marked reduction in polymorphonuclear neutrophils and metamyelocytes and a slight increase in the proportion of myelocytes, promyelocytes and myeloblasts in the marrow of the deficient animals. The leukocyte-erythroid ratio was decreased. In addition, extremely immature nucleated red cells were present which differed from the basophilic normoblasts seen in the marrow of normal pigs and in those fed diets low in protein or deficient in iron. These cells were large, measuring 12 to 15 microns in diameter, and their nuclear chromatin was delicate and meshlike. In some cells the chromatin showed a tendency to clump, in others the chromatin appeared finely granular and more homogeneous. A delicate nuclear membrane separated the relatively large nucleus from the homogeneous basophilic cytoplasm. The more immature of these cells contained two or three distinct nucleoli. Later stages were present, including the orthochromatic stage. This entire series of cells constituted about 15 per cent (3 to 40) of all the bone marrow cells. Photomicrographs were included in the previous report.<sup>1</sup>

These cells resembled closely the megaloblasts seen in the marrow of patients with pernicious anemia, the only distinct difference being that the nuclear chromatin was not as fine and meshlike as that seen in megaloblasts in man. Whether

or not the cells described are pig megaloblasts is a matter for conjecture but for purposes of discussion in this paper these cells will be referred to as megaloblasts in order to distinguish them from the immature red cells (normoblasts) seen in other types of anemia in swine. It is noteworthy that these megaloblasts were present in the bone marrow in the same proportion in group D as in group A, B and C, in spite of the administration of liver extract from the beginning of the experiment.

*Tyrosyl, Allantoin and Uric Acid Excretion* The data on the urinary excretion of tyrosyl, allantoin and uric acid, both before and one month after pteroylglutamic acid therapy, are summarized in table 4. These represent the means of three consecutive daily determinations in each of 5 animals.

The term tyrosyl is used to refer collectively to the hydroxyphenyl compounds (tyrosine, p-hydroxyphenyllactic acid and p-hydroxyphenylpyruvic acid) as determined by the method of Folin and Ciocalteu.<sup>26</sup> No significant change was noted in the excretion of tyrosyl. Determinations were done daily on 2 animals for

TABLE 4—*Studies on the Urinary Excretion of Tyrosyl, Allantoin and Uric Acid in Pigs Before and After Pteroylglutamic Acid Therapy*

Determination	Number Pigs	Deficient	Treated
Tyrosyl mg	5	6.7 $\pm$ 1.38	8.0 $\pm$ 1.69
Allantoin mg	5	10.4 $\pm$ 1.37	7.8 $\pm$ 0.91
Uric Acid mg	5	6.1 $\pm$ 0.84	5.5 $\pm$ 1.10
Allantoin + Uric Acid mg	5	16.5 $\pm$ 1.68	13.3 $\pm$ 1.71

Results are expressed in mg./kilo body weight/24 hours

thirty days following therapy. No consistent increase or decrease was noted either within the first day or two, or later. The amount excreted per day by a single animal varied from day to day as much as 575 mg.

The 24 hour urinary excretion of allantoin and uric acid was not significantly different before therapy as compared with the excretion one month later. However, immediately following therapy, in association with the reticulocytosis, there was a marked increase in the excretion of allantoin as shown in figure 3. A similar increase in uric acid excretion did not occur.

*Pathologic Studies\** Autopsy material including sternal, rib and femoral bone marrow, liver, spleen, stomach, kidney, lung, skeletal muscle and cardiac muscle, was obtained from 7 untreated animals in which a deficiency of pteroylglutamic acid had been produced. The bone marrows showed a striking cellular hyperplasia. Megakaryocytes were present in approximately normal numbers except in the marrow of pig 10-88. This animal had a marked thrombocytopenia (72,000 per cu mm) just prior to death, and the number of megakaryocytes was reduced.

Microscopic examination of the liver, spleen, kidneys, lungs and cardiac muscle failed to reveal any significant abnormalities although small areas of interstitial

\* We are indebted to Dr. F. D. Gunn, Professor of Pathology, University of Utah, for these studies.

hemorrhage were seen in sections of the lungs of two animals. An increase in hemosiderin was not observed in any of the organs studied. Areas of atrophy, hyalinization and segmental necrosis were present in the sections of skeletal muscle. Similar changes have been observed in the muscles of animals fed a diet low in protein.

*Response to Various Therapeutic Agents* The increases in reticulocytes and volume of packed red cells following therapy with pteroylglutamic acid compounds (23 animals) are summarized in table 5. Representative examples are illustrated in detail in figures 4, 5, 6, 7 and 8. In every instance except two, a reticulocytosis of

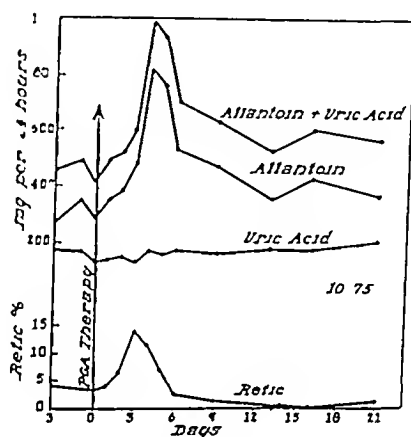


FIG. 3.—Urinary excretion of uric acid and allantoin (fig 10-75 Group B) following a single intramuscular injection of 10 mg of pteroylglutamic acid. Note the marked increase in the excretion of allantoin during the period of reticulocytosis. The excretion of uric acid remained relatively constant.

11 per cent or greater followed therapy with a pteroylglutamate. In half of the animals treated there was a reticulocytosis of 21 to 42 per cent. The reticulocyte curve rose sharply with a peak on either the third or fourth day following therapy, as illustrated in figure 4, in all but two instances. In these the peak was reached on the second and fifth days, respectively. Simultaneously with or just following the reticulocytosis there occurred a rapid rise in the volume of packed red cells and a return to normal or near normal values within two to three weeks. Frequently the increase was as great as 10 to 15 ml/100 ml in the first week following therapy (figure 4). In all instances except two the increase was greater than 10 ml/100 ml and in one-half of the animals the rise was greater than 15 ml/100 ml. The mean corpuscular volume returned to normal more slowly and frequently macrocytosis persisted in the absence of anemia.

An initial leukocyte increase, generally greater than 5000 per cu mm, invariably followed this type of therapy (table 8). After several weeks the leukocytes then decreased somewhat but in 20 of the 23 animals so treated the values for total leukocytes were sustained within the normal range. A thrombocytosis also oc-

curred in almost every instance but due to the great variability in the number of platelets the results are difficult to interpret

In the bone marrow pteroylglutamic acid therapy was associated with a reversion to normal. There was a decrease in the megaloblast-like cells, an increase in the myeloid-erythroid ratio and a shift to the right in the myeloid series. Frequently large masses of platelets, covering several low-power fields, were present in the bone marrow preparations.

TABLE 5—Results of Therapy with Pteroylglutamic Acid Compounds

Pig Number	Compound	Dose mg I.M.	Before Therapy		After Therapy			
			V.P.R.C. ml /100 ml	Retic %	Retic Peak		V.P.R.C.	
					%	Day	ml /100 ml	Day
10-53	PGA	100	23	3	15	4	41	12
10-54	PGA	20	26	1	15	3	40	26
10-56	PGA	20	27	1	6	4	41	12
10-64	PGA	20	21	1	24	3	37	12
10-72	PGA	20	25	2	33	3	43	32
10-73	PGA	20	28	1	16	3	45	22
10-75	PGA	10	30	3	14	3	43	16
10-81	PGA	0.1	23	4	16	4	33	9
10-83	PGA	20	29	2	11	4	43	13
10-84	PGA	20	25	2	17	3	33	27
10-85	PGA	0.05	29	1	7	4	31	14
10-92	PGA	20	20	2	34	4	44	7
10-93	PGA	20	21	3	35	4	40	26
10-95	PGA	20	9	3	25	3	35	20
10-96	PGA	20	23	2	17	3	36	18
10-99	PGA	20	21	2	28	3	36	20
10-97	PDGA	50	23	2	42	3	40	21
10-74	PTGA	20	37	3	21	3	49	42
10-77	PTGA	20	27	3	16	3	42	21
10-78	PTGA	20	26	1	13	3	39	14
10-98	PTGA	20	20	2	23	2	41	6
10-85	PHGA	20	23	1	34	4	40	32
10-96	PHGA	20	13	2	45	5	29	18

PGA pteroylglutamic acid

PDGA, pteroyldiglutamic acid

PTGA pteroyltriglutamic acid

PHGA pteroylheptaglutamic acid

V.P.R.C. volume of packed red cells

Pteroyldiglutamic acid (figure 4), pteroyltriglutamic acid (figure 8) and pteroylheptaglutamic acid were as effective as pteroylglutamic acid (table 5) in restoring the blood and bone marrow to normal. The one animal (10-97) treated with the diglutamate, one (10-98) of the four animals treated with the triglutamate and both animals (10-85, 10-96) treated with the heptaglutamate were fed the purified casein diet and so presumably had received little extrinsic factor.

The effects of therapy with various commercial liver extracts and with vitamin

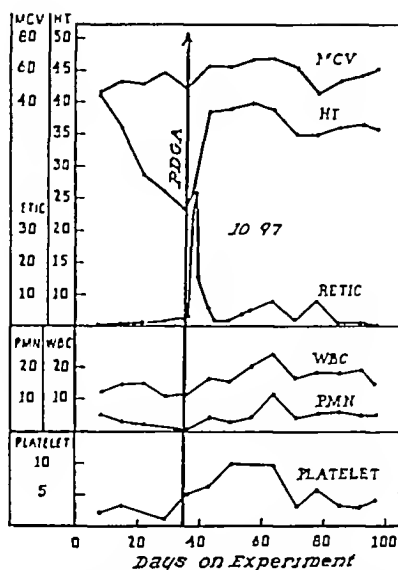


FIG 4.—Fig 10-97 (Group C purified casein, 10 per cent) Note the marked reticulocytosis and increase in volume of packed red cells (Ht), leukocytes (WBC) and polymorphonuclear cells (PMN) following the intramuscular administration of 50 mg of pteroyldiglutamic acid

MCV refers to mean corpuscular volume in  $\mu$ , Ht refers to volume of packed red cells, ml/100 ml retic refers to reticulocytes, per cent WBC refers to total leukocyte count thousands per c mm PMN refers to polymorphonuclear cells thousands per c mm, Plat refers to platelets times 100 000 per c mm

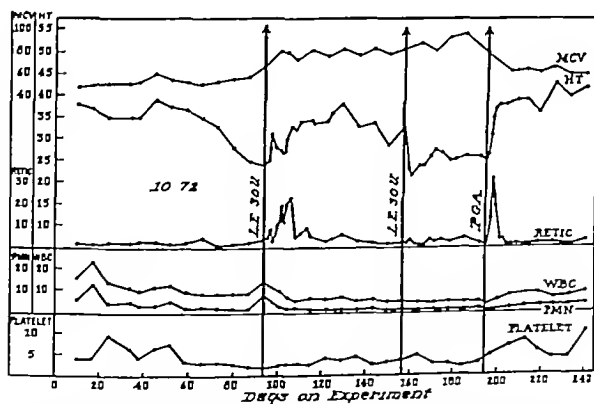


FIG 5.—Fig 10-72 (Group B, crude casein, 26 per cent) Note the reticulocytosis and gradual increase in the volume of packed red cells (Ht) following the intramuscular administration of 2 ml (No 1039 30 USP units) of purified liver extract This represents the greatest response to liver extract observed Compare with figures 7 and 8 After becoming anemic again this animal failed to respond to a second injection of liver extract (No 1039 30 USP units) although it responded promptly to 20 mg of pteroyldiglutamic acid intramuscularly It is noteworthy that this animal was receiving 26 per cent crude casein and consequently had available liberal amounts of extrinsic factor For symbols see figure 4



B<sub>12</sub> are presented in table 6. Representative examples are illustrated in figures 5, 6, 7, 8. A significant reticulocytosis of more than 10 per cent occurred in 33 per cent

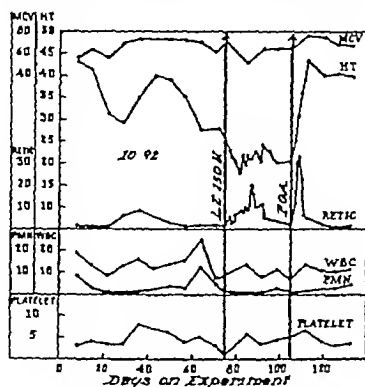


FIG. 6—Pig 10-92 (Group D, purified casein 10 per cent plus 15 U S P units of purified liver extract (No. 1039, 15  $\mu$ /ml) every 15 days). This animal developed anemia, leukopenia, neutropenia, and thrombocytopenia in spite of the administration of 15 units of purified liver extract every 15 days from the beginning of the experiment. The intramuscular administration of 10 ml (150 units) of the same liver extract in a single injection was followed by a reticulocytosis but there was no increase in volume of packed red cells (Ht.). Twenty mg of pteroylglutamic acid (intramuscularly) produced a prompt response in reticulocytes and volume of packed red cells. For symbols see figure 4.

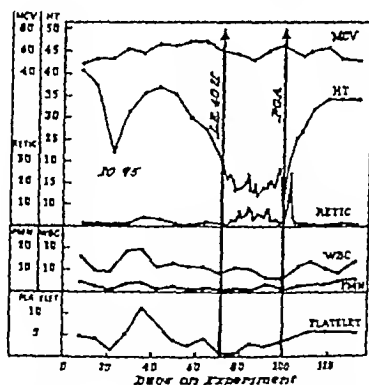


FIG. 7—Pig 10-95 (Group C, purified casein 10 per cent). Following the intramuscular administration of 2 ml of purified liver extract (No. 1124, 40 units) there was a slight reticulocytosis but no increase in the volume of packed red cells (Ht.). This animal failed to respond to liver extract in spite of the fact that he was given purified casein (extrinsic factor poor). A rapid response took place following the administration of 20 mg of pteroylglutamic acid intramuscularly. For symbols see figure 4.

(5 out of 15) of the trials whereas a similar response occurred in 91 per cent (21 out of 23) of the therapeutic trials with the pteroylglutamates. The reticulocyte curve in general rose gradually, was flat in shape and the peak, if present, was delayed

(figures 5, 6, 7) as compared with the peaks following pteroylglutamic acid therapy (figures 4, 5). The reticulocyte response to liver extracts was no greater in the

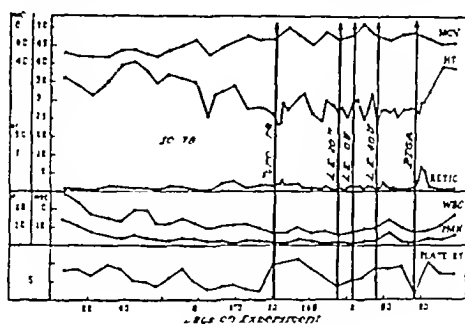


FIG. 8—Fig 10-78 (Group A, crude casein 10 per cent) Note the failure to respond to tyrosine liver extract (No. 1124, 20 units followed by 60 units and No. 1067, 40 units) and the subsequent response to a single intramuscular injection of 20 mg of pteroylglutamic acid. For symbols see figure 4.

TABLE 6—Results of Therapy With Liver Extracts and Vitamin B<sub>12</sub>

Pig Number	Liver Extract			Before Therapy		After Therapy			
	Number	USP Units/ml	Total USP Units	V P R C ml/100 ml	Retic %	Retic Peak		V P R C	
						%	Day	ml/100 ml	Day
10-53	1039	15	150	24	4	5	2	23	12
10-54	1039	15	75	18	1	15	4	26	20
10-56	1039	15	15	15	1	5	7	27	24
10-72	1039	15	30	24	3	24	12	38	35
10-81	1039	15	75	25	4	4	8	23	13
10-85	1039	15	75	27	3	4	5	31	14
10-90	1039	15	30	29	2	4	4	34	33
10-92	1039	15	150	21	2	21	12	20	30
10-73	1124	20	80	31	4	11	10	34	10
10-78	1124	20	80	27	2	3	6	27	23
10-95	1124	20	40	20	2	9	12	9	28
10-99	1124	20	40	21	1	10	9	21	33
10-77	1067	4	40	25	3	14	4	38	17
10-78	1067	4	40	25	1	5	4	27	20
10-80	1067	4	40	30	1	5	6	38	10
10-76	1063	0	0	29	2	4	6	33	9
10-81	1066	0	0	25	1	5	8	23	21
10-96	Vitamin B <sub>12</sub>		125 µg	20	1	6	2	31	29

All liver extracts given intramuscularly in a single injection

V P R C. refers to volume of packed red cells.

Liver extract 1039 Parke Davis and Co. 1124 Armour and Co. 20 mg solids per ml. 1067 Armour and Co. 1063 Armour and Co. containing *in vitro* factors of Hays (43). 1066 Armour and Co. one ml derived from 46 grams of fresh liver.

animals given the purified casein diet than in the animals fed crude casein. There was no correlation between the number of units of liver extract given and the degree of response. The greatest reticulocytosis observed following liver extract therapy

was 24 per cent (pig 10-72, figure 5) After becoming anemic a second time, this animal then failed to respond to a second injection of liver extract but responded promptly to 20 mg of pteroylglutamic acid Pig 10-92 (figure 6) was given the purified casein diet and 15 U S P units of liver extract every two weeks from the beginning of the experiment When severe anemia developed, 10 ml of liver extract (150 units/ml) was administered in a single injection In spite of the previous administration of liver extract, reticulocytosis appeared which reached a peak of 21 per cent on the 12th day following therapy However, the reticulocyte

TABLE 7—Results of Therapy With Various Other Substances

Pig Number	Therapy				Before Therapy		After Therapy			
	Compound	Route	Dose	Days	V P R C ml /100 ml	Retic %	Retic Peak		V P R C	
							%	Day	ml /100ml	Day
10-76	Thymine	Oral	10 Gm	5	26	1	3	6	26	13
10-77	Thymine	Oral	10 Gm	7	24	1	5	4	27	16
10-81	Thymine	Oral	10 Gm	3	23	3	21	7	34	11
10-81	Thymine	Oral	10 Gm.	5	34	1	7	8	32	35
10-77	Uracil	Oral	10 Gm	5	27	3	5	2	27	17
10-77	Adenine	Oral	5 Gm	5	27	3	5	5	24	14
10-80	Xanthopterin	I.M.	20 mg	1	30	3	9	10	34	20
10-80	Xanthopterin	Oral	25 mg	5	32	1	2	6	30	21
10-78	Tyrosine	Oral	10 Gm.	10	26	3	5	5	27	35

V P R C. refers to volume of packed red cells.

TABLE 8—The Initial Rise in the Leukocytes Following Pteroylglutamic Acid Therapy (23 pigs) as Compared With Liver Extract Therapy (17 pigs)

Initial Rise in WBC $\times 1000/c$ mm	Pteroylglutamic Acid Compounds No Pigs	Liver Extracts No Pigs
0	0	3
1-5	4	10
5-10	12	2
10-15	7	0

curve was irregular and was not accompanied by a rise in volume of packed red cells

A significant increase in the volume of packed red cells following liver therapy was observed in only 5 pigs (10-54, 10-56, 10-72, 10-77, 10-80) and in these the rise was 8, 12, 14, 13 and 8 ml /100 ml respectively By comparison, pteroyl glutamic acid therapy was followed by a rise in the volume of packed red cells greater than 10 ml /100 ml in 91 per cent of the trials The rise was prompt as well as marked In general, the mean corpuscular volume was little affected by liver extract therapy (figures 6, 7, 8)

An initial increase in leukocytes following liver therapy occurred in 12 out of 15 trials but it was not as marked as the rise following pteroylglutamic acid therapy (table 8) In 10 instances the leukocyte level was sustained within the normal

range Thrombocyte increases occurred irregularly but in general the increases were not sustained

The changes in the bone marrow which followed liver therapy were of the same type and degree as the changes noted in the peripheral blood. In most instances, as the number of leukocytes rose following the injection of liver, there was an increase in metamyelocytes and neutrophils and an increase toward normal in the leukocyte-erythroid ratio. In the bone marrow of those animals in which the anemia responded partially to liver therapy there was a reduction in the relative numbers of megaloblast-like cells but in no instance did they disappear entirely. If no response to liver was observed, no changes were noted in the bone marrow.

Crystalline vitamin B<sub>12</sub> was administered to one pig (table 6). The reticulocytes rose from 1 to 6 per cent and the volume of packed red cells increased from 20 to 31 in 29 days.

The results of therapy with various other substances are presented in table 7. Uracil, adenine and tyrosine were inactive in the doses given. Xanthopterin in a single injection of 20 mg (1 mg/kilogram of body weight) produced, in a single animal, a slight reticulocytosis of 9 per cent and a small unsustained rise in volume of packed red cells. This was then followed by 25 mg (1.9 mg/kilogram) of xanthopterin daily by mouth for five days without a further response. A response to thymine with a reticulocytosis of 21 per cent was observed in one animal (10-81). Although the volume of packed red cells, leukocytes and platelets increased, the increase was not maintained and a second course of thymine was ineffective. In two other pigs (10-76, 10-77) no response followed the administration of thymine.

#### DISCUSSION

Macrocytic anemia, leukopenia due to a proportionately greater reduction in polymorphonuclear than in mononuclear cells, slight thrombocytopenia, and a bone marrow picture showing erythroid hyperplasia and immature red cells resembling the megaloblasts of pernicious anemia, developed in swine fed low or high levels of crude or purified casein and given sulfasuxidine and a pteroylglutamic acid antagonist. A clear cut and sharp hemopoietic response was observed in such animals whenever pteroylglutamic acid was given. The administration of liver extract, however, was associated with only a slight effect. Thus, in 10 out of 15 trials, no or only slight activity was observed. In 5 trials a significant reticulocytosis occurred but the reticulocyte peak was delayed and the curve was flat as compared with that produced by pteroylglutamic acid. Although an increase in volume of packed red cells followed liver extract therapy in 5 pigs, this increase was delayed, submaximal and unsustained.

These blood and bone marrow changes developed in the experimental animals not only when crude casein containing extrinsic factor was fed, but even in pigs injected every 15 days with liver extract containing 15 units of anti-pernicious anemia principle. Thus it seems clear that a hematologic syndrome with morphologic characteristics similar to those of pernicious anemia can be produced experimentally in the pig in the presence of the anti-pernicious anemia liver factor. This condition would appear to be similar to the pteroylglutamic acid-responsive, liver-

refractory megaloblastic anemias described in human subjects, namely, tropical macrocytic anemia,<sup>44</sup> <sup>45</sup> <sup>46</sup> macrocytic anemia of pregnancy,<sup>47</sup> achrestic anemia,<sup>48</sup> and refractory megaloblastic anemia<sup>49</sup>

It is not likely that the response to liver extract, such as it was, can be attributed to the pteroylglutamic acid content of the extracts. More than 50  $\mu\text{g}$  of pteroylglutamic acid has been found to be required by the pig in order to elicit a significant response (table 6). The extracts used were found by assay with *Lactobacillus casei* in our laboratory as well as by others<sup>20</sup> to contain only about 1 to 5  $\mu\text{g}$  of the vitamin per ml, and the greatest response to liver extract (pig 10-72) occurred following the injection of only 2 ml. Furthermore, there was no correlation between the number of ml of extract given and the degree of response. Again, extracts containing no anti-pernicious anemia activity (nos. 1063 and 1066) but possessing the same quantities of pteroylglutamic acid were ineffective. The less highly purified extract (no. 1067, 4 units/ml) containing about 5  $\mu\text{g}$  of pteroylglutamic acid per ml was no more effective in the pig than the more highly refined extract (no. 1124, 20 units/ml) which contained only about 1  $\mu\text{g}$  per ml.

Since the response following the administration of crystalline vitamin  $\text{B}_{12}$ \* was of the same type as that following purified liver extract, it is reasonable to assume that the effectiveness of the liver extracts was due to their vitamin  $\text{B}_{12}$  content (or to a chemically related substance) rather than to a third factor and that in those pigs responding to liver extract there existed a partial deficiency of the liver factor in addition to the pteroylglutamic acid deficiency. Consistent with this is the observation that pig 10-72 (fig. 5) failed to respond to a second injection of liver extract after becoming anemic a second time. Presumably the deficiency had been satisfied by the injection of liver extract and a second response was therefore not obtainable.

Animals receiving crude casein containing considerable extrinsic factor activity responded as well to liver extract as did those receiving purified casein. It must be concluded, therefore, that a partial deficiency of liver principle developed in spite of the availability of extrinsic factor. On the other hand, pigs fed a diet similar in all respects to the low protein diet used in these experiments, with the exception that a pteroylglutamic acid antagonist was not included, did not respond at all to liver extract.<sup>51</sup> This suggests that in pteroylglutamic acid deficiency the requirement for a factor in liver extract (vitamin  $\text{B}_{12}$ ) is increased. One may speculate whether pteroylglutamic acid plays a role in the release, absorption or synthesis of vitamin  $\text{B}_{12}$  in the intestinal tract.

The hypothesis has been presented<sup>52</sup> that pteroylglutamic acid functions in some way in the synthesis of thymine and that  $\text{B}_{12}$  serves as a coenzyme which is concerned with the conversion of thymine to thymidine. According to this hypothesis, the curative effects of pteroylglutamic acid in pernicious anemia depend upon increased thymine synthesis, which, by mass action, leads to the formation of thymidine. The effectiveness of large amounts of thymine in pernicious anemia is explained on a similar mass action hypothesis. If this view is correct, then pigs

\* This finding has been confirmed in five additional pigs.

deficient in pteroylglutamic acid should respond to large doses of thymine. Such was not the case.

The pteroylglutamic acid deficient swine, receiving either crude or purified casein, responded rapidly and maximally to each of the three pteroylglutamic acid conjugates tested. This indicates that in these animals there was adequate utilization of the conjugates. If the animals were actually in a state of liver factor depletion, as suggested by assays of the livers of similar animals,<sup>11</sup> then it is difficult to accept the hypothesis that the liver factor is necessary for the proper utilization of the naturally occurring conjugates.<sup>52</sup> However, it must be admitted that it is much easier to find flaws in current hypotheses concerning the role of pteroylglutamic acid and the anti-pernicious anemia factor in metabolism than it is to offer an explanation which is wholly satisfactory.

Swendseid, Wandruff and Bethell<sup>54</sup> have found that the urinary excretion of total phenols and hydroxyphenyl acids is increased in patients with pernicious anemia in relapse and that within twenty-four hours following therapy with liver extract there is a marked reduction in the phenolic fraction containing the hydroxyphenolic acids. It has also been claimed that liver suspensions from pteroylglutamic acid deficient rats are better able to oxidize tyrosine after the addition of pteroylglutamic acid than in the absence of this substance<sup>55</sup> and that either pteroylglutamic acid<sup>56</sup> or anti-pernicious anemia liver extracts<sup>57</sup> are capable of reducing the increased keto acid and tyrosyl excretion in scorbutic guinea pigs. The results of the tyrosyl excretion studies presented here fail to indicate the presence of a defect in tyrosine metabolism in pigs.

The markedly increased excretion of allantoin in the urine during the period of reticulocytosis following therapy with pteroylglutamic acid is similar to the increase in uric acid excretion which occurs in patients with pernicious anemia following therapy with liver.<sup>58</sup> However, since a similar increased excretion takes place during the regenerative phase following hemorrhage,<sup>59</sup> it is likely that this merely represents increased hemopoietic activity and a rapid turnover of nucleic acids in the bone marrow.

#### SUMMARY

1 A deficiency of pteroylglutamic acid has been produced in 32 swine fed a purified diet containing casein and supplemented with seven B vitamins, sulfasuxidine and a folic acid antagonist. The casein was fed at two levels, 10 and 26 per cent. Two types of casein were used: a crude preparation possessing significant extrinsic factor activity and a purified casein with little activity.

2 The hematologic manifestations observed were (a) severe macrocytic anemia, (b) leukopenia, due to a proportionately greater reduction in polymorphonuclear than in mononuclear cells, (3) slight thrombocytopenia, and (4) hyperplastic bone marrow with an increase in immature nucleated red cells which resemble the megaloblasts seen in the bone marrow of patients with pernicious anemia.

3 The feeding of a 26 per cent rather than a 10 per cent crude casein diet did not prevent but did delay the onset of the blood changes. Anemia developed most rapidly in the animals receiving 10 per cent purified casein.

4 The group receiving 26 per cent casein developed a greater degree of macrocytosis in the same period of time than did the group receiving 10 per cent casein. In all groups the degree of macrocytosis increased as the duration of the anemia increased.

5 The hematologic manifestations were not delayed nor was their development prevented by the intramuscular administration of 15 U S P units of liver extract every 15 days.

6 The blood and bone marrow returned rapidly to normal following the administration of pteroylglutamic acid, pteroyldiglutamic acid, pteroyltriglutamic acid and pteroylheptaglutamic acid. Thymine and xanthopterin had little or no activity. Tyrosine, adenine and uracil were inactive.

7 Purified liver extracts and crystalline vitamin B<sub>12</sub> were found to possess some hemopoietic activity in several animals but the activity was considerably less than that of the pteroylglutamic acid compounds.

8 The urinary excretion of tyrosyl (hydroxyphenyl compounds) was not abnormal in the pteroylglutamic acid deficient pigs and was not altered by either pteroylglutamic acid or liver extract therapy.

9 The urinary excretion of allantoin and uric acid did not differ significantly from the normal. Immediately following therapy with pteroylglutamic acid, however, in association with the reticulocytosis and lasting for the same period, there was a marked increase in the excretion of allantoin.

10 The results suggest that both pteroylglutamic acid and a factor in liver extract similar to or identical with vitamin B<sub>12</sub> are required for normal hemopoiesis in the pig.

#### ACKNOWLEDGEMENTS

The crude methylfolic acid antagonist xanthopterin and the pteroylglutamic acid compounds, with the exception of pteroylheptaglutamic acid, were kindly furnished by the Lederle Laboratories, Pearl River, New York, through the courtesy of Dr. T. H. Jukes and Dr. S. M. Hardy.

Sulfasuxidine was generously furnished by Sharp & Dohme, Inc., Philadelphia, Pa., through the courtesy of Dr. W. A. Feiler.

Pteroylheptaglutamic acid and Natola were supplied by Parke, Davis & Company, Detroit, Mich., through the courtesy of Dr. A. E. Sharp and Dr. J. J. Pflieger.

Biotin was obtained from Hoffmann-La Roche, Inc., Nutley, N. J., through the courtesy of Dr. E. L. Severinghaus.

The vitamins, with the exception of pteroylglutamic acid and biotin and including vitamin B<sub>12</sub>, were kindly furnished by Merck and Company, Inc., Rahway, N. J., through the courtesy of Dr. A. Gibson and the late Dr. D. F. Robertson.

Experimental liver extracts (No. 1124, 1063, 1066 and 1067) were generously furnished by Armour and Company, Chicago, Illinois, through the courtesy of Dr. E. E. Hays.

We are indebted to Mrs. Darlene Kehl, Mr. George Trappett and Mr. Otis Hadley for technical assistance.

#### REFERENCES

- <sup>1</sup> CARTWRIGHT, G. E., FAY, J., TATTING, B. AND WINTROBE, M. M. Pteroylglutamic acid deficiency in swine. Effects of treatment with pteroylglutamic acid, liver extract and protein. *J. Lab. & Clin. Med.* 33: 397, 1948.
- <sup>2</sup> MINOT, G. R. AND MURPHY, W. P. A diet rich in liver in the treatment of pernicious anemia. *J. A. M. A.* 89: 759, 1927.

- <sup>2</sup> CASTLE, W. B. Observations on the etiologic relationship of achylia gastrica to pernicious anemia. I. The effect of the administration to patients with pernicious anemia of the contents of normal human stomach recovered after the ingestion of beef muscle. *Am J M Sc* 178: 748 1929.
- <sup>4</sup> MILLER, D. K. AND RHODES, C. P. The experimental production of loss of hematopoietic elements of the gastric secretion and of the liver in swine with achlorhydria and anemia. *J Clin Investigation* 14: 153 1935.
- <sup>5</sup> WINTROBE, M. M., SAMTER, M. AND LISCO, H. Morphologic changes in the blood of pigs associated with deficiency of water soluble vitamins and other substances contained in yeast. *Bull Johns Hopkins Hosp* 64: 399, 1939.
- <sup>6</sup> SMITH, S. G., REISER, R. AND HARRELL, G. T. Spontaneous recovery from nutritional macrocytic anemia in young swine following initial estrus. *J Clin Investigation* 20: 369 1941.
- <sup>7</sup> MCGOWAN, J. P. AND SINCLAIR, R. D. A further method of estimating the potency of liver extract. *Edinburgh M J* 38: 405 1931.
- <sup>8</sup> LAWRASON, F. D., AND CROCKITE, E. P. Incidental finding of megaloblast like cells in bone marrow of one of two swine with macrocytic anemia and achlorhydria. Naval Medical Research Institute, December 1947, Project NM007039 report No. 1.
- <sup>9</sup> WELCH, A. D., HEINLE, R. W., SHARPE, G., GEORGE, W. L. AND EPSTEIN, M. Chemical antagonism of pteroylglutamic acid in a pig: hematopoietic effect of extrinsic and intrinsic factors. *Proc Soc Exper Biol & Med* 65: 364 1947.
- <sup>10</sup> HEINLE, R. W., WELCH, A. D., GEORGE, W. L., EPSTEIN, M. AND PRITCHARD, J. A. Effect of extrinsic factor, liver extract and folic acid on induced macrocytic anemia in swine. *J Lab & Clin Med* 32: 1398 1947.
- <sup>11</sup> WELCH, A. D., HEINLE, R. W., PRITCHARD, J. A. AND SALIS, H. Influence of pteroylglutamic acid on the synthesis and action of the antipernicious anemia factor. *Fed Proc* 7: 300 1948.
- <sup>12</sup> WILLS, L. AND STEWART, A. Experimental anemia in monkeys with special reference to macrocytic nutritional anemia. *Brit J Exp Path* 16: 444 1935.
- <sup>13</sup> — AND BILIMORIA, H. S. Studies in pernicious anemia of pregnancy. V. Production of a macrocytic anemia in monkeys by deficient feeding. *Indian J Med Research* 20: 391 1932.
- <sup>14</sup> —, CLUTTERBUCK, P. W. AND EVANS, B. D. F. A new factor in the production and cure of certain macrocytic anemias. *Lancet* 1: 311 1937.
- <sup>15</sup> — AND —. A new factor in the production and cure of macrocytic anemias and its relation to other haematopoietic principles curative in pernicious anemia. *Biochem J* 31: 2136 1937.
- <sup>16</sup> RHODES, C. P., AND MILLER, D. K. The production in dogs of chronic black tongue with anemia. *J Exp Med* 58: 585 1933.
- <sup>17</sup> — AND —. The association of Bartonella bodies with induced anemia in the dog. *J Exp Med* 61: 139 1935.
- <sup>18</sup> HANDLER, P. AND FEATHERSTON, W. P. The biochemical defect in nicotinic acid deficiency. II. On the nature of the anemia. *J Biol Chem* 151: 395 1943.
- <sup>19</sup> RUEGAMER, W. R., BRICKSON, W. L., TORBET, N. J., AND ELVERHJEM, C. A. Response of dogs to liver extracts containing the pernicious anemia factor. *J Nutrition* 36: 425 1948.
- <sup>20</sup> DAVIS, J. E. The experimental production of a hyperchromic anemia in dogs which is responsive to anti-pernicious anemia treatment. *Am J Physiol* 142: 402, 1944.
- <sup>21</sup> —. Hyperchromic anemia produced by choline or acetylcholine and the induced remission of both by folic acid or liver injections. The probable mechanism of action of liver and folic acid in treatment of anemia. *Am J Physiol* 147: 404 1946.
- <sup>22</sup> KUNKEL, A., KROP, S., AND WESCOE, W. C. Effect of folic acid and liver extract on serum and red cell cholinesterase activity. *Am J Physiol* 152: 309 1948.
- <sup>23</sup> WATSON, G. M., CAMERON, D. G. AND WITTS, L. J. Experimental macrocytic anemia in the rat. *Lancet* 2: 404 1948.
- <sup>24</sup> BENNETTS, H. W. AND BECK, A. B. Enzootic ataxia and copper deficiency of sheep in Western Australia. Bulletin No. 147, Council for Scientific and Industrial Research. Commonwealth of Australia Melbourne, 1942.
- <sup>25</sup> —, —, HARLEY, R., AND EVANS, S. T. Falling Disease of cattle in the south west of Western Australia. 2. Studies of copper deficiency in cattle. *Australian Vet J* 27: 85 1941.



- <sup>6</sup> Quoted in CARTWRIGHT G E Dietary factors concerned in erythropoiesis *Blood* 2 111 and 256 1947
- <sup>7</sup> CAMPBELL, C J McCABE, M M. BROWN R. A, AND EMMETT A D Crystalline vitamin B<sub>12</sub> in relation to the cellular elements of chick blood *Am J Physiol* 144 348 1944
- <sup>8</sup> FRANKLIN A L. STOKSTAD E L R. BELT, M AND JUKES, T H Biochemical experiments with a synthetic preparation having an action antagonistic to that of pteroylglutamic acid *J Biol Chem* 169 427 1947
- <sup>9</sup> TOTTER, J R. Vitamin M deficiency *Ann New York Acad Sc* 48 309 1946
- <sup>10</sup> STOKSTAD E. L. R. AND JUKES, T H Absence of appreciable L cas i factor effect in anti pernicious anemia liver extract *Proc Soc Exper Biol & Med* 62 112, 1946
- <sup>11</sup> BUSABARGER R. A IVY, A C WIGODSKY H S AND GUNN F D The effect of gastrectomy on the monkey *Ann Int Med* 13 1018 1939
- <sup>12</sup> PETRI S AND JENSEN H Experimental studies on production of pernicious anemia by operation on the digestive tract *Acta med Scandinav* 106 274 1941
- <sup>13</sup> — BANG O KIAER, W AND NIELSEN A K. Experimental studies on the significance of the various regions of the stomach to the antipernicious-anemic principle content of the liver (in swine) II Counter-effect of nicotinic acid on the disappearance of antipernicious-anemic principle in the liver following resection of the fundus of the stomach *Acta med Scandinav* 116 273 1944
- <sup>14</sup> WINTROBE M M. MILLER J L. AND LISCO H The relation of diet to the occurrence of ataxia and degeneration in the nervous system of pigs *Bull Johns Hopkins Hosp* 67 377 1940
- <sup>15</sup> FRANKLIN A L. STOKSTAD E L R. AND JUKES T H Acceleration of pteroylglutamic acid deficiency in mice and chicks by a chemical antagonist *Proc Soc. Exper Biol & Med* 65 368 1947
- <sup>16</sup> FOLIN O AND CIOCALTEU V On tyrosine and tryptophane determinations in proteins *J Biol Chem* 73 627 1927
- <sup>17</sup> MEDES G A new error of tyrosin metabolism tyrosinosis The intermediary metabolism of tyrosine and phenylalanine *Biochem J* 26 917 1932
- <sup>18</sup> LEVINE S Z MARPLES E AND GORDON H H A defect on the metabolism of tyrosine and phenyl alanine in premature infants I Identification and assay of intermediary products *J Clin Investigation* 29 199 1941
- <sup>19</sup> YOUNG E G AND CONWAY C F On the estimation of allantoin by the Rimini-Schryver reaction *J Biol Chem* 142 839 1942
- <sup>20</sup> FORSHAM P H THORN G W PRUNTY F T AND HILLS A G Clinical studies with pituitary adrenocorticotropin *J Clin Endocrinology* 8 15 1948
- <sup>21</sup> KERN A AND STRANSKY E. Beitrag zur kolorimetrischen Bestimmung der Harnsäure *Biochem Ztschr* 290 419 1937
- <sup>22</sup> WINTROBE M M Nutritive requirements of young pigs *Am J Physiol* 126 375 1939
- <sup>23</sup> HAYS E E A method for studying the effect of various substances upon red cell maturation *in vivo* *Am J M. Sc* 216 528 1948
- <sup>24</sup> WILLS, L. Pernicious anemia nutritional macrocytic anemia and tropical sprue *Blood* 3 36 1948
- <sup>25</sup> GOODALL, J W D GOODALL, H I AND BANERJEE D Folic acid in nutritional anaemia *Lancet* 1 20 1948
- <sup>26</sup> KEMP T A Liver and folic acid in the treatment of nutritional macrocytic anaemia *Lancet* 2 350 1947
- <sup>27</sup> DAVIDSON L S P GIRDWOOD R H AND CLARK J R Pernicious anaemia of pregnancy and puerperium. *Brit M J* 1 819 1948
- <sup>28</sup> WILKINSON J F Folic acid *Brit M. J* 1 771 1948
- <sup>29</sup> DAVIDSON L S P Refractory megaloblastic anemia *Blood* 3 107 1948
- <sup>30</sup> DART F S Pteroylglutamic acid ( folic acid ) liver extract, and amino acids in the treatment of granulocytopenia in rats *Public Health Reports U.S.P.H.S* 62 1785 1947
- <sup>31</sup> CARTWRIGHT G E TATTING B AND WINTROBE M M Niacin deficiency anemia in swine *Arch Biochem* 19 109 1948
- <sup>32</sup> WRIGHT L D SKEGGS H R AND HUFF J W The ability of thymidine to replace vitamin B<sub>12</sub> as a growth factor for certain Lactobacilli *J Biol Chem* 175 475, 1948

- <sup>11</sup> WELCH A D The present status of pteroylglutamic acid and of other hematopoietic agents Fed Proc 6 471, 1947
- <sup>12</sup> SWENDSEID M E WANDRUFF B AND BITHIELL F H Urinary phenols in pernicious anemia J Lab & Clin Med 3 1242, 1947
- <sup>13</sup> RODNEY G SWENDSEID M E AND SWANSON A L Tyrosine oxidation by livers from rats with a sulfasuxidine induced pteroylglutamic acid deficiency J Biol Chem 168 395 1947
- <sup>14</sup> WOODRUFF C W AND DARTY W J An *in vivo* effect of pteroylglutamic acid upon tyrosine metabolism in the scorbutic guinea pig J Biol Chem 172 851, 1948
- <sup>15</sup> SEALOCK R R AND LEPOW J P Antipernicious anemia extracts and tyrosine metabolism in the scorbutic guinea pig J Biol Chem 174 763 1948
- <sup>16</sup> RIDDLE M C Endogenous uric acid metabolism in pernicious anemia J Clin Investigation 8 69 1929
- <sup>17</sup> KRATTA J, JR Endogenous uric acid and hematopoiesis II Uric acid, reticulocytes and erythrocytes after hemolysis by phenylhydrazine hydrochloride J Biol Chem 86 223, 1930

# THE USE OF REPLACEMENT TRANSFUSION IN DISEASES OTHER THAN HEMOLYTIC DISEASE OF THE NEWBORN

By M. BESSIS, M.D.

## INTRODUCTION AND HISTORY

**R**EPLACEMENT transfusion is an operation which combines blood letting and transfusion at the same time and in the same patient. The idea of using this technic is very old, it is interesting to recall that the first physicians who tried transfusions in the seventeenth century began by doing replacement transfusions. Eutyphronus remarked, "It is foolish to transfuse a patient without previous blood letting, because this would not reduce the strain on the body."

This operation, although it had a few successes, caused quite a few deaths. This is easily understood since the transfusions were usually done with animal blood. After the report of Claude Perrault to the Academy of Science in Paris in 1664, this body declared the transfusion a dangerous method. In 1675, Parliament passed a law prohibiting its use.

Since the discovery of blood groups by Landsteiner, the use of transfusion has increased greatly, but replacement transfusion was almost completely abandoned. At most, it was used in a few cases of carbon monoxide poisoning, mushroom poisoning, and intensive burns. Even in those cases, only one blood letting and a transfusion of 500 to 1000 cc. was performed. The purpose of this article is to discuss not replacement transfusion as it was used then, but the replacement of the total blood volume of a patient by the blood of many donors and the repetition of that technic many times in the same patient.

The progressive realization of a complete blood replacement in man was achieved in 1946. We had thought for some time that such an operation would be of great value if it was well tolerated by the patient. In 1939-1940 we studied with our director, A. Tzanck, and our associate, M. Burstein, a technic for rapid replacement transfusion in the dog. We showed that in that animal the total blood volume could be replaced by a mixture of fresh blood of other dogs without any serious reaction. We achieved thus not a simple blood letting followed by transfusion, but a true "washing out" of the organism. At the same time, we attempted similar results in man, but did not succeed completely. After the war, in 1945-1946, studies on the Rh factor and hemolytic disease of the newborn gave a new impetus to this problem.

It is known that in hemolytic disease of the newborn the infant has in his body both Rh positive blood cells and anti-Rh serum. A few persons thought that the most rational treatment would be replacement transfusion of the newborn. That operation was proposed and carried out by Wallerstein, Wiener, Bessis, and others. A further advance was realized with the method of Diamond who uses a plastic

From the Research Laboratory of the National Center of Blood Transfusion, Paris, France

catheter which is pushed up to the main vessels and which enables total replacements\* to be done under ideal conditions

In spite of the successes of replacement transfusion in the newborn, the same operation had not, to our knowledge, been used in adults until 1947. The recent progress in immunology following discovery of the Rh factor and similar blood groups and of the conditions under which a person can have irregular agglutinins, led us to attempt such an operation. Although there was a possibility that repeated transfusions might cause some reactions of incompatibility due to rare or still unknown antigens, the fact that the majority of those accidents are due to Rh or similar factors which can be prevented by careful selection of donors justified our attempt.

There was also the possibility of reactions due to intolerance on the part of the recipient or transmitted by the injected blood, accidents whose possible frequency is multiplied by the number of donors. This is the reason why our first experiences were performed on a very limited scale and only on patients suffering from incurable disease and in a moribund state. Little by little, however, we have attempted more complete transfusions. Led by an hypothesis which we will discuss later, we tried total replacement transfusion in a child suffering from acute leukemia. This operation was successful and proved thereby both the innocuousness of the replacement transfusion and its action in leukemia.

Our experience, which is based on over 190 replacement transfusions† in children and adults, has confirmed the first proof, because we have had no serious accident. Moreover, in all these cases the general condition of the patient was clearly and rapidly improved. We shall discuss later the precautions which must be observed in the choice of blood to be injected, and our results. We do not wish to say that the fear which we had before trying the procedure is unjustifiable, because an accident is always possible.

The results which we report in this article are not of equal value. Many patients, treated in the early period of trial, received only one replacement transfusion. The operation was not repeated either because we did not know then that one replacement transfusion was insufficient or because we had difficulty in getting blood of the proper group or because of absence of suitable veins. As an example, we can take the first case of acute anuric nephritis in which we did only one replacement transfusion and obtained no results. It was only with experience that the necessity for repeating the replacement transfusion at intervals varying with the condition of the patient became evident and that we obtained consistently good results.

\* By total replacement we mean replacement of 85 to 95 per cent of the blood volume, and this is done by replacement transfusion of two to three times the patient's blood volume. (This is explained more fully in the section on "Technic.")

† These include, cases of acute anuric nephritis, acute leukemia, chronic leukemia, lipid nephrosis, generalized carcinoma, severe icterus, myeloma, lymphosarcoma, acute polyarthritis, and acute hypertensive nephritis.

## I TECHNIC OF REPLACEMENT TRANSFUSION IN THE CHILD AND IN THE ADULT

We will give here the principle of the method, and those interested in technical details may refer to the work of S. Buhot. The drawing and injection of the blood are done at the same time so that the total volume is unchanged. In these conditions the percentage of the transfused blood in the organism as compared to the quantity injected is as follows (after Wiener and Wexler)

<i>Quantity of blood injected</i>	<i>Percentage of blood transfused in the patient's organism</i>
$\frac{1}{2}$ volume of patient's blood	39.4
1 volume of patient's blood	63.2
$1\frac{1}{2}$ volumes of patient's blood	77.7
2 volumes of patient's blood	86.5
$2\frac{1}{2}$ volumes of patient's blood	91.8
3 volumes of patient's blood	95.0

The first problem is to find the necessary quantity of blood for the replacement transfusion. For an adult who has an average blood volume of 5 liters, we need 15 liters, usually obtained from 30 donors, and these must be of the same ABO and Rh groups. If we cannot get sufficient blood of the proper A or B group, we use O group blood after neutralizing the anti-A and anti-B agglutinins with Wittebsky's AB substances.

We use fresh blood collected in bottles containing citrate solution. Our experience has shown that such transfusions are well tolerated by the patient and give no serious reactions if we give calcium intravenously to prevent tetany caused by the fixation of the blood calcium to the sodium citrate. Lately we have modified our technic and have used heparin, 2 mg per kilo of body weight, since the clotting time of the patient during the operation is so lowered as to render the drawing of blood very difficult.

We draw the blood either from a vein of the elbow on the side opposite to the injection or from the femoral vein. We use vacuum bottles to obtain a rapid flow of blood. However, the easiest method is to use a plastic catheter as suggested by Diamond for replacement transfusion in the newborn, and to introduce it in a superficial vein either after cutdown or through a large bore needle, pushing the tip up to one of the larger veins. It is then easy to draw the desired amount of blood and to inject by the same route. The rapid flow of blood in the large veins prevents us from withdrawing the blood which we have just injected. The catheter also spares the patient the inconvenience of the pressure cuff which is very painful after a time.

Lately we have simplified the operation by the use of the electrical pump of Dausser and Moulinier which is essentially a plastic pump electrically driven and with a reversible action. One end is connected to the plastic catheter and the other to the donor's blood flask and to the used blood receptacle. The pump draws the blood from the patient at any desired speed, e.g., 300 cc. in 5 minutes. The flow is then reversed and the pump is used to transfuse the patient with donor's blood. This operation is repeated until the desired number of liters has been given. Only two persons are needed for the whole procedure, which includes the drawing of the

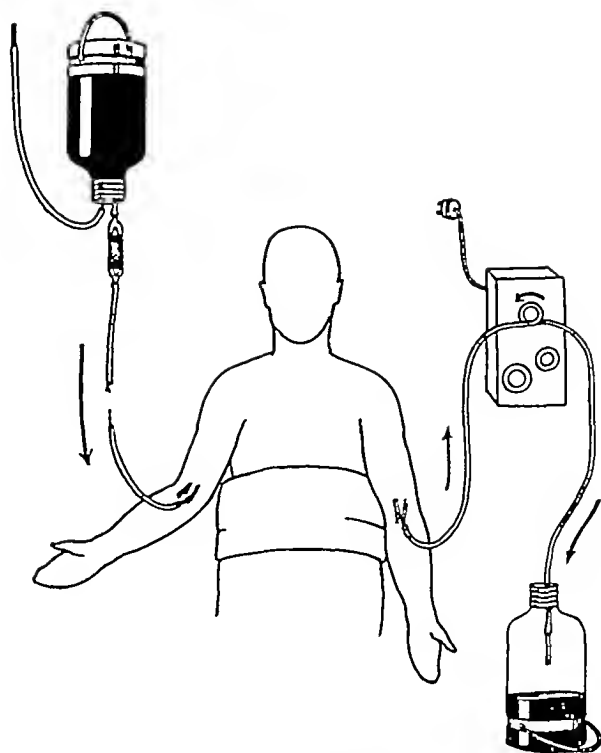


FIG. 1.—EXSANGUINO TRANSFUSION PERFORMED IN A HUMAN ADULT

The injection is done on one side the bleeding on the other. The same route can be used for both injection and bleeding by using the special pump of Dausset and Moulinier.

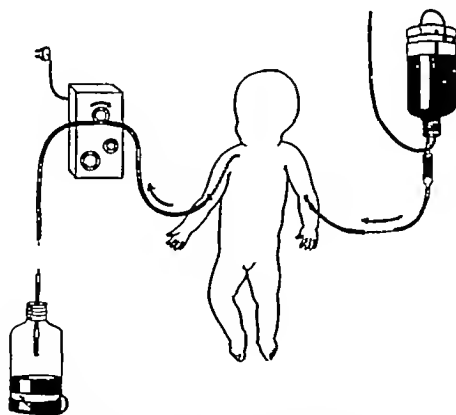


FIG. 2.—EXSANGUINO-TRANSFUSION PERFORMED ON A CHILD

Bleeding is done with the help of a catheter (Diamond) so as to reach a large blood vessel. Same remark as for figure 1.

blood from the donors. We have thus been able to draw from the patient and to transfuse 16 liters of blood in 3 hours. The operation lasts from one hour in the child to two to four hours in the adult.

The reactions we encountered are of little gravity—chills and urticaria—but they are more frequent than with the usual transfusions (30% of the cases). This may be due to the fact that our material was not checked for pyrogenic substances. Sometimes we have seen a temperature rise which lasted one to two days.

## II. TREATMENT OF CERTAIN INTOXICATIONS AND ANURIC NEPHRITIS BY REPEATED REPLACEMENT TRANSFUSIONS

The indication for replacement transfusion is evident in the course of an intoxication when the toxic product is in the blood, for example, in hemolytic disease of the newborn where the antibodies and the coated red cells are circulating in the serum. Other examples are cases of severe intoxication due to benzol, potassium chlorate, etc. But replacement transfusion is also indicated when the toxic product is produced by the organism itself and is found in the blood. By this we mean hemoglobinemia and other products of hemolysis whatever may be the cause—septicemia, hemolytic poisons, crush syndrome, and the transfusion reactions due to incorrect grouping or typing. In all these conditions replacement transfusion combats the anemia and, what is more important, replaces the pathological plasma with normal plasma, thus preventing or diminishing the secondary renal reactions.

However, we think that the most important indication for replacement transfusion is in anuric nephritis.\* In these cases the kidneys, although they have been subjected to a great insult, are capable of regaining their previous morphologic and physiologic status. This is supported by the postmortem findings of anatomical lesions in various stages of repair. Thus we have the impression that if those patients could have survived a few days the disease would have tended to end favorably. In these cases, replacement transfusion, by withdrawing with the patient's blood the toxic products contained in it and replacing this blood with normal blood, plays the role of eliminatory organ and allows the survival during the time necessary for the kidneys to regain their normal function. We have observed that replacement transfusions of moderate size (5 liters), repeated every second or third day† withdraw sufficient urea (25 Gm. from a patient whose urea blood level is 500 mg. per cent) and other toxic products to enable survival of the patient until the return to normal of the kidney function. The records of the patients treated in this manner have already been published. We will mention here one of the observations.

\* These replacement transfusions have evidently no resemblance to the operation described by Carrel and Joltrain under the name of washing the blood which consists of withdrawing small quantities of the patient's blood, washing the red cells in normal saline and reinjecting the washed blood into the patient.

† Some persons have questioned whether the withdrawal of blood has any effect on the N urea level. We have noted, as can be seen by our charts, that the first replacement transfusion does not change the urea level. This is proof that the urea of the tissues has diffused in the normal blood injected. After several replacement transfusions, however, the abnormal urea of the tissues is slowly lowered and the blood urea level tends to return to normal.

## CASE REPORT\*

A patient 26 years old entered the hospital February 18 1948 following an intentional abortion with the clinical findings of a septicemia due to *B. perfringens* which was confirmed by blood culture February 19. She was treated by massive doses of penicillin. On February 20 the RBC was 1 620 000 per cc. and her serum was strongly icteric, as was her skin. She was in marked oliguria. A replacement transfusion of 8 liters was given and considerably improved her general condition. The icterus disappeared in twelve hours. The RBC the next day was 4 070 000. The serum was of a normal color, the toxic products of acute hemolysis having been eliminated. There appeared slight purpura and the patient was in severe oliguria with a urea level of 250 mg per cent. This oliguria persisted for twenty two days. During that time we performed five replacement transfusions of 4 to 6 liters each, withdrawing each time 15 to 25 Gm of urea and other toxic products. These operations were well supported by the patient, and normal diuresis was gradually regained. The urea level fell slowly until it was normal on April 1.

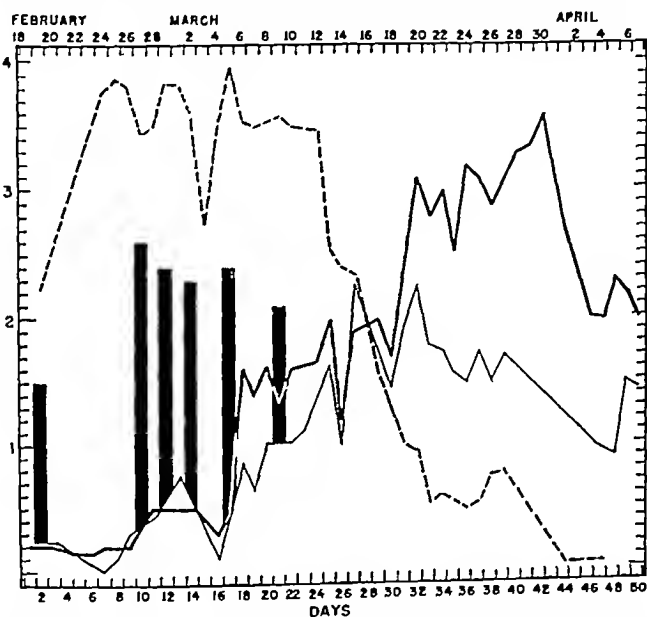


FIG. 3.—Evolution of blood urea (dots 1 = 1 Gm), volume of urines (thick line 1 = 1 liter) urea eliminated per day (fine line 1 = 10 Gm) the principle observation of anuric nephritis treated by exsanguino transfusion. The black columns indicate the urea amount drawn out by successive exsanguinations (1 = 10 Gm) (observation made by P. V. Ravor and M. Millicez and co-workers).

This observation shows that (1) Replacement transfusion is able to transform a person suffering from uremia from a preagonal to a normal condition in a few hours. (2) This operation removes the greater part of the toxic products of acute hemolysis and, if done before the anuria sets in, possibly diminishes the secondary renal complications. (3) Repeated massive replacement transfusions can, for a short time, act like the kidneys, thus allowing survival until kidney function is regained. (4) The urea concentration in the urine remains very low in spite of the

\* Pasteur Vallery Rador, Millicez and colleagues.



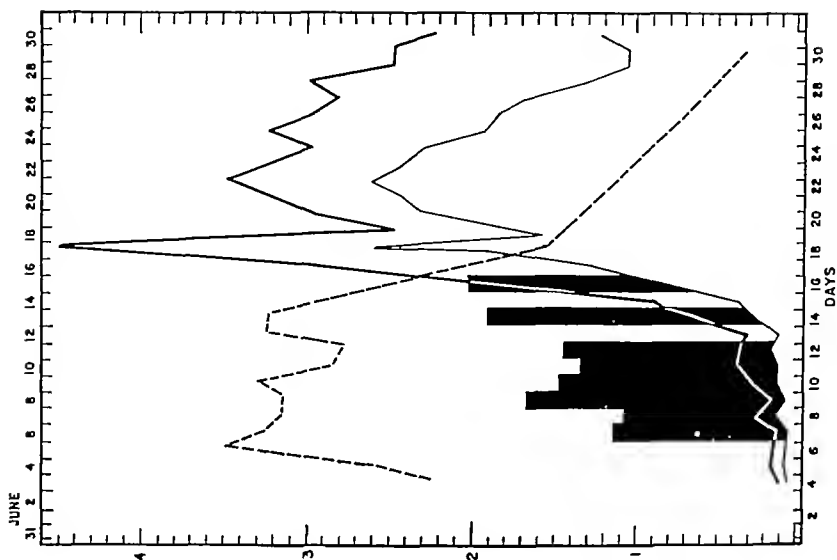


FIG. 4.—Evolution of Acute Nephritis Treated Successfully by Exchanging Transfusion Same symbols as figure 3 (Patient of A. Tzanck and J. Dausset)

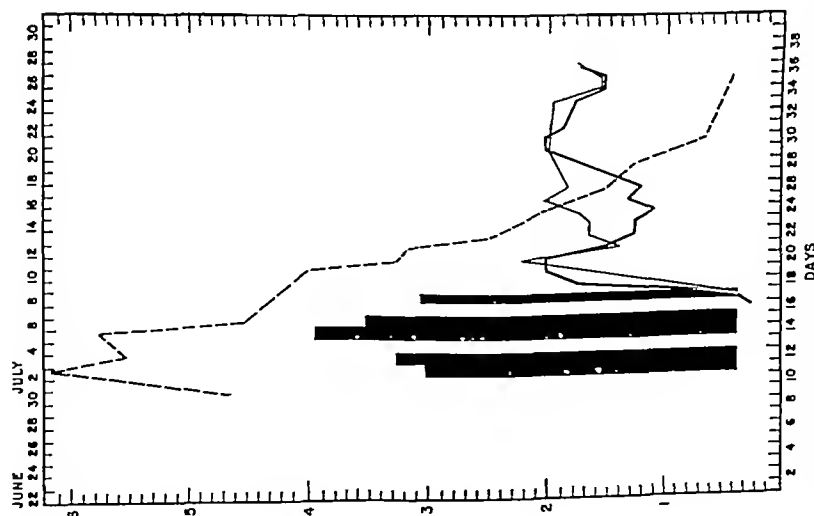


FIG. 5.—Same remark as figure 4

return to normal of the urea level of the blood, and this fact, added to the numerous observations of patients cured by dialysis, confirms the opinion that the kidney lesions regress very slowly. On the average it takes three months for complete recuperation. Dr. Dausset has treated 6 other cases of anuric nephritis of which three had been treated previously or alternatively by intraperitoneal dialysis and were in a moribund state. The 6 cases survived. The data are given in figures 4-5.

A comparative chart of the effects of the replacement transfusion as compared to those of intraperitoneal dialysis follows:

Replacement Transfusion	Intraperitoneal Dialysis
1. Removes all the toxic products including the nondialyzable ones such as hemoglobin, myoglobin, stromatol	1. Removes only the dialyzable toxic products
2. Does not modify the normal equilibrium of the tissue fluids	2. Unless special precautions are taken, normal equilibrium is destroyed either by adding or taking away too much electrolytes or adding too much water which may lead to cerebral edema
3. Does not cause any severe reactions	3. Usually causes peritonitis either of the plastic type by the formation of adhesions or, in certain cases, the infectious type
4. Can be used as often as needed	4. Possibility of peritonitis prevents its continuous use for more than a few days and frequently prevents its reuse
5. Is very efficient: removes a larger quantity of toxic products which can be calculated beforehand	5. Removes a lesser quantity which cannot be calculated beforehand
6. Painless and rapid	6. Inconvenient and slow

### III REMISSIONS IN ACUTE LEUKEMIA TREATED BY REPLACEMENT TRANSFUSIONS

The principle behind the use of replacement transfusion in leukemia is based on the hypothesis that there is an antileukemic substance in normal blood. This hypothesis is based on the good results which have been occasionally noted after ordinary transfusions. Clinical and hematologic remissions in leukemia after transfusion have always been rare, but they can not be denied, as was reported by Dreyfus. In addition to those complete remissions, cases of clinical and peripheral blood improvement have been frequently reported after transfusion. However, no one paid much attention to these remissions, and Wintrobe, in his *Clinical Hematology* (1947 edition), says in brief that transfusions in leukemia can be used against the anoxemia and the bleeding, and that in one case he had noticed a remission of a few months' duration, which however could not be repeated. He goes on to say that in view of the expense and trouble and temporary effect, there are few indications for transfusion in acute leukemia.

We believe that we have proven in the 38 cases which we have treated that, contrary to what has been reported after single transfusions, total or partial remissions

(2) In 30 cases, we witnessed in succeeding days a clinical remission consisting in the disappearance of adenopathy, hepatosplenomegaly, temperature, etc. In 15, these clinical remissions were accompanied by the return to normal of the peripheral blood and an amelioration of the marrow, and in 6 of these there was complete clinical, peripheral blood, and marrow remission (3) The remission lasted in general three weeks to three and one-half months. However, of the patients with complete remission are still alive after eleven months, or in complete remission, the other in clinical remission. The other four complete remissions lasted one to three and one half months. When a patient relapsed, the clinical



FIG. 9.—Sternal puncture of same patient as in figure 8 but after a series of frequently repeated exsanguino transfusions (Observation made by Fagart and Angibeaux. *Rev. Hemat.* 4, 1948)

remission was less marked, although due to diverse reasons transfusion was not fully used in all the patients.

These results have been duplicated in a few other centers in France. Though not by no means perfect, this technic brings some hope to the leukemics and indicates a new approach to the problem of acute leukemia.

#### IV POSSIBLE INDICATIONS OF REPLACEMENT TRANSFUSIONS IN MEDICAL RESEARCH

To bring to the attention of other persons interested in research and the diseases the possibilities of this technic, we shall examine it rapidly for its usefulness in (a) withdrawing toxic products from the organism, (b) injecting in physiologic quantities the important substances which a normal person has in his



FIG. 7—Bone marrow puncture done on same patient as in figure 6 but after three exsanguino transfusions

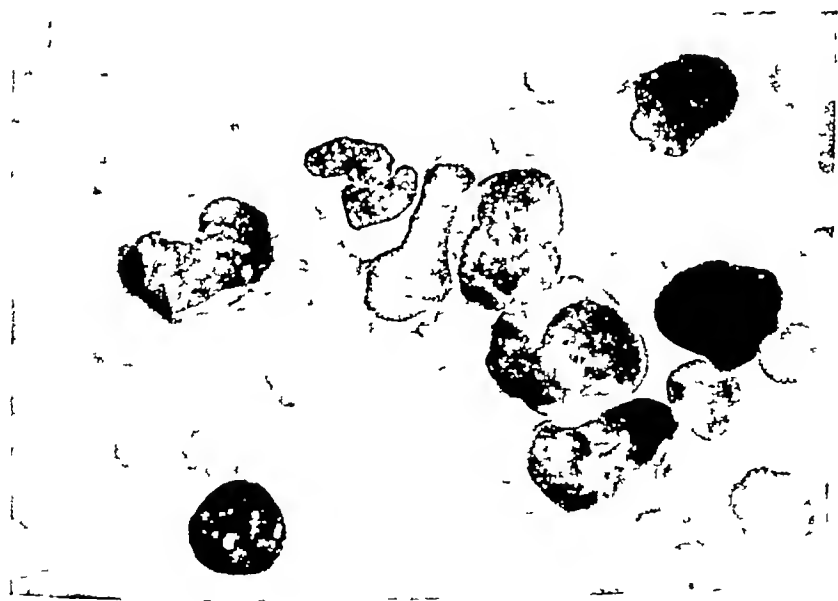


FIG. 8—Sternal puncture performed on a patient with acute leukemia

(2) In 30 cases, we witnessed in succeeding days a clinical remission consisting of the disappearance of adenopathy, hepatosplenomegaly, temperature, pain, bleeding. In 15, these clinical remissions were accompanied by the return to normal of the peripheral blood and an amelioration of the marrow, and in 6 of these there was complete clinical, peripheral blood, and marrow remission (3) The remission lasted in general three weeks to three and one-half months. However, 2 of our patients with complete remission are still alive after eleven months, one in complete remission, the other in clinical remission. The other four complete remissions lasted one to three and one-half months. When a patient relapsed, the effect of re-



FIG. 9.—Sternal puncture of same patient as in figure 8 but after a series of frequently repeated short exsanguine transfusions (Observation made by Fagart and Angibaux. *Rev. Hemat.* 4, 1948.)

placement transfusion was less marked, although due to diverse reasons transfusion was not fully used in all the patients.

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#### IV POSSIBLE INDICATIONS OF REPLACEMENT TRANSFUSIONS IN MEDICAL RESEARCH

To bring to the attention of other persons interested in research and blood diseases the possibilities of this technic, we shall examine it rapidly for its usefulness in (a) withdrawing toxic products from the organism, (b) injecting in physiologic quantities the important substances which a normal person has in his blood.

and which are lacking in sick persons, (c) studying the evolution of a disease in a subject whose blood has returned to normal, (d) realizing better a condition of parabiosis between a patient and a healthy donor

(a) *The replacement transfusions enable the withdrawal of toxic substances* This fact is evident when the substance is known and can be calculated in the blood. As we have shown already, they are just as useful when we are dealing with a toxic substance which is in the blood and also in the rest of the organism. Of course, this does not hold in the cases where the toxins are fixed irreversibly on tissues other than the blood. In the case of diffusible toxins, by changing the blood of the patient we remove a small part of that poison, but as a new state of equilibrium is obtained between the poisons in the organism and the fresh blood injected, the repeated removal of the blood will enable us to remove completely the toxin from the organism. It would be very interesting to know if other toxins, known or theoretic, could be thus withdrawn from the organism, i. e., radioactive substances or their secondary disintegration products, and thus prevent the medullary aplasia which they cause. In the same line of thought it could be used against acute benzol poisoning.

This technic could also be used to study certain diseases due to as yet unidentified auto-antibodies of the serum, i. e., certain hemolytic anemias, certain types of nephritis. Just as it can replace renal function, this technic could possibly be used to replace the liver function in cases of severe icterus. In general we think that it could be used successfully in all the reversible pathologic conditions in which the main condition for the survival of the patient is that we keep him alive a few days until the organism returns to normal.

(b) *A total substitution of blood enables us to inject in physiologic quantities known and unknown substances* (1) We would like to point out that in many cases where immuno-transfusions have not given the expected results, it has been due to the small quantities used and that in certain cases it was theoretically impossible to hope for any result. On the other hand, we do think that it would be worthwhile if the total blood volume of the patient were replaced by the blood of an immunized donor, and if this operation were repeated many times, the patient would receive a large quantity of antibodies. (2) In many diseases certain substances are absent from the patient's blood and it seems evident that replacement transfusions, especially if repeated, would correct that lack. And if the correction of the lack is noted after the replacement transfusion, it might provide a clue to identifying the cause of the disease.

(c) *Replacement transfusion permits us to study the evolution of a disease with a normal blood* Thus it gives us a means of studying that part of a disease which is due to its action on the tissue cells and that which is due to its modifications of the constituents of the blood or the plasma. It also gives us a means to study the manner and time of evolution of a disease once we have brought back the blood to normal. For example, in a case of lipoid nephrosis if we bring the blood back to normal with replacement transfusions, we can then watch the same disease picture reappear. This technic can also be used to study the survival of red cells, white cells, and platelets.

(d) *Replacement transfusions enable us to realize the condition of parabiosis in man*

Many research groups undoubtedly have had the idea that it would be very interesting to join the circulation of two persons, one sick and one healthy, in order to study the modifications which would be caused in both. Such an operation, however, in practice is impossible in all diseases in which we are not absolutely sure that they are nontransmittable. It would be very important to know what would happen when a patient suffering from a disease of unknown etiology is put in direct circulation with a healthy person. We can use the example of a case of leukemia. There are three possibilities (1) both persons would become leukemic, (2) the leukemic person remains leukemic and the normal person remains normal, (3) the leukemic person returns to normal. This example would also apply to cases of cancer, chronic rheumatism, etc.

As we have already said, such an experiment may be impossible, but repeated replacement transfusions enable us, if not to obtain completely the state of a crossed circulation, at least to approximate it very closely. Of course, we lose all the results on the normal person. However, we can get those results that occur in the sick patient. We could thus find for any disease whether normal blood protects a person by hormones, or antibodies, or other substances which it carries, or whether in certain diseases it has no role at all.

#### SUMMARY

The technic of exchange transfusion in adults and children is given. It differs from that in newborns only by the use of arm or leg veins and of a motor driven pump to withdraw and inject the blood. The use of exchange transfusion in acute toxemia with anuria was tried on the theory that by withdrawing sufficient toxic products, the patient could be tided over the acute phase. Seven patients were thus treated, all with success.

The use of exchange transfusion in leukemia is based on the theory that normal persons have an antileukemic substance in their blood. Thirty-eight cases were treated with the following results: 30 clinical remissions, of which 15 also had peripheral blood remissions, and of these, 6 had complete clinical peripheral blood and marrow remissions. The author concludes by pointing out some possible applications of this technic.

#### REFERENCES

- BESSIS M. L'exsanguino transfusion en dehors de la maladie hémolytique du nouveau-né. Acquisition Médicales récentes 1 vol Paris Flammarion Ed 1948
- , Indications de la substitution sanguine par exsanguino-transfusion. Paris M d No 24 304-308 1948
- , AND BERNARD, J. Indications de l'exsanguino-transfusion en dehors de la maladie hémolytique du nouveau-né. Sang 19 40-45 1948
- , AND —. A propos du traitement de la leucémie aigue par exsanguino-transfusion. Bull Acad Méd 131 615 1947
- DAUSSET J. AND MOULINIER J. Une nouvelle pompe électrique permettant l'exsanguino-transfusion. Sang 8 538 1948
- DEROT M. TANRET P. AND DAUSSET J. Unpublished observations
- PASTEUR VALÉRY RADOT, MILLIEZ, P., LAROCHE, CL., LHERMITTE F. AND LEVASSEUR Ch. Première observation de néphrite aigue azotémique indiscutablement guérie par la méthode de Bessis. Bull et Mem Soc. Med Hôp 64 908-915, 1948

- REVOL, L. L'exsanguino-transfusion dans les leucémies aiguës Communication aux journées internationales d'Hématologie, in press (Paris)
- ROBERT, J. Le traitement des leucoses aiguës en particulier par l'exsanguino-transfusion Thèse Lyon, 1 vol Lyon Imprimerie des Beaux Arts 1948 87 pages
- TZANCK, A. BESSIS M. AND BURSTEIN, M. Recherches sur le remplacement du sang circulant par du sang frais, du sang conserve ou du plasma Compt rend Acad Sci 22. 822-824, 1946
- AND DAUSSET, J. L'exsanguino-transfusion dans les anuries Bull et Mem Soc Méd des Hôp 64. 563-569 1948



# NITROGEN MUSTARD THERAPY IN HODGKIN'S DISEASE

## ANALYSIS OF FIFTY CONSECUTIVE CASES

By WILLIAM DAMESHEK, M D , LOUIS WEISFUSE, M D , AND TOBIAS STEIN, M D

### INTRODUCTION

MUSTARD gas was first discovered by Ritchie in 1854 and prepared for manufacture by Meyer in 1886. It was first used as a war gas by the Germans at Ypres in the spring of 1915. Five hundred deaths and 14,276 casualties resulted from this initial attack. By the end of the war, there was a total of 400,000 casualties from mustard gas poisoning. The clinical course of these victims was described by Marshall,<sup>1</sup> Mandell and Gibson<sup>2</sup> and others. Pappenheimer and Vance,<sup>3</sup> Warthin and Weller,<sup>4</sup> Lynch et al.<sup>5</sup> studied the effects of mustard gas upon experimental animals. Krumbhaar and Krumbhaar<sup>6</sup> reported upon the hematologic complications.

With the advent of World War II, the Chemical Warfare Service of the United States Army undertook a systematic study of the mustard gases as potential offensive agents. In 1940, these chemicals were submitted, among others, to Drs. L. Goodman and A. Gilman, then at the Yale Medical School, for pharmacologic evaluation. During the course of their investigations they found that following the parenteral administration of an aqueous solution of nitrogen mustard in normal rats, there developed a marked lymphocytopenia together with some degree of anemia and thrombocytopenia. Dr. Thomas Dougherty of the Yale Department of Anatomy studied the effects of the chemical in the spontaneous leukemia and lymphosarcoma of rats. In a number of cases, a marked reduction in the size of abnormal tissues took place.

The possibility then suggested itself that nitrogen mustard might be of some value in the treatment of the leukemias and lymphomata of man. The first patient, a terminal case of lymphosarcoma, was treated at the New Haven Hospital in August 1941 with a dramatic regression of involved glands. One of us (W. D.) was requested to examine the experimental and clinical data obtained in these preliminary studies. Further trial with other patients seemed desirable and a supply of the chemical was given to us for this purpose.

After an initial period terminating with the close of the war, nitrogen mustard was distributed under the auspices of the National Research Council to observers in various parts of the country. Such a cooperative program has made possible a rapid and thorough clinical evaluation of the nitrogen mustards. The historical background, as well as the chemical, pharmacologic, toxicologic, and experi-

From the Ziskind Laboratories (Hematology Section) of the New England Centre Hospital, the U. S. Veterans Hospital, West Roxbury, Massachusetts and the Department of Medicine, Tufts College Medical School, Boston, Mass.

Aided by grants from Merck and Company, Inc., the Charlton Fund and the American Cancer Society (Massachusetts Division).

mental aspects have been reviewed by Gilman and Philips<sup>7</sup> and the initial clinical results were described by Jacobson<sup>8</sup> and Goodman et al.<sup>9</sup> Favorable results have been reported in Hodgkin's disease, lymphosarcoma, leukemia, polycythemia vera,<sup>10-19</sup> mycosis fungoides<sup>20-23</sup> and Boeck's sarcoid.<sup>24</sup> The general results obtained by 120 cooperating physicians are currently being analyzed by Dr. David A. Karnofsky at the Memorial Hospital in New York. Tentative detailed analyses have already been submitted for review.<sup>25-26</sup> Nitrogen mustard has been found to be ineffective in carcinomata (except carcinoma of the lung), Ewing's sarcoma, melanosarcoma and neuroblastoma. The general results obtained have recently been summarized.<sup>27</sup>

Our work with  $\text{HN}_2$  was begun in 1942. We were early impressed with its favorable effects in Hodgkin's disease and in certain cases of lymphosarcoma, although our results with leukemia were disappointing. As our studies continued,

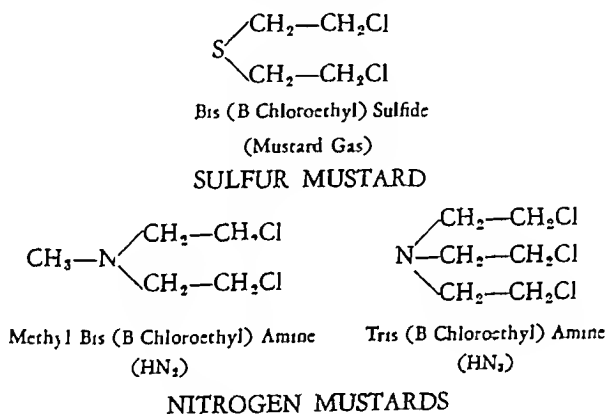


FIG. 1.—CHEMICAL FORMULAE OF SULFUR AND TWO NITROGEN MUSTARDS

the often remarkable therapeutic effects of  $\text{HN}_2$  in Hodgkin's disease became more and more apparent. The present paper deals with a study of the effects of the drug in our first 50 consecutively treated cases of Hodgkin's disease. For the most part, these were moderately and far advanced, oftentimes terminal cases, presenting constitutional symptoms in addition to their local disease.

#### THEORETICAL CONSIDERATIONS

The chemical formulae of the sulfur and nitrogen mustards are shown in Figure 1. Dichloroethyl sulfide is the formula for mustard gas. The most widely used nitrogen mustard is methyl bis (B-chloroethyl) amine, subsequently abbreviated as  $\text{HN}_2$ . In tris (B-chloroethyl) amine, or  $\text{HN}_3$ , the methyl group is replaced by a third chloroethyl group. The sulfur mustards are soluble only in oils whereas the nitrogen mustards are readily soluble in water.

In aqueous solutions, the nitrogen mustards undergo intramolecular cyclization<sup>7</sup> (figure 2). Gilman and Philips<sup>7</sup> have shown that the imino ring possesses an unusual reactivity. It reacts with a great variety of biologically important

groups, i e , alpha amino, sulfhydryl, phenolic, carboxyl, imidazole, imino, inorganic phosphates, chick pepsin peptodase, choline oxidase, etc In the presence of chloride ion, the reaction tends to reverse itself with reformation of the parent amine <sup>28</sup> This probably occurs in the extracellular fluids where the concentration of chloride ion is high Entrance of the parent amine into the cell where there is little, if any, chloride ion to compete with water, results in a rapid transformation with intramolecular cyclization and alkylation of labile groups The speed of this reaction was demonstrated by Karnofsky et al <sup>29</sup> By occluding the circulation to the femoral bone marrow and the small intestine for periods ranging from 2 to 5 minutes, these organs were completely protected from the generalized leukoroxic action of the nitrogen mustards

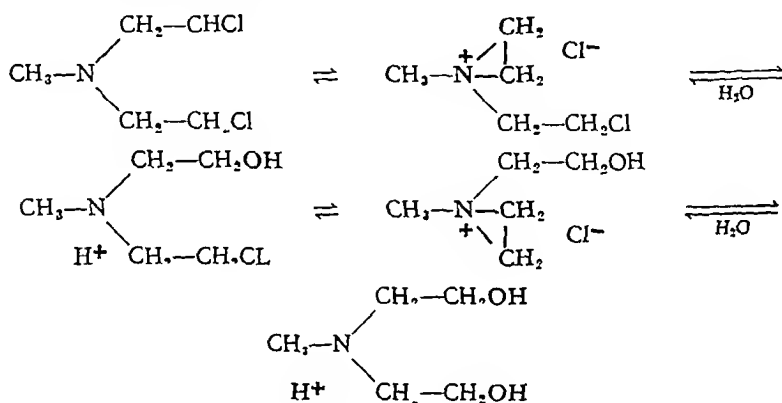


FIG 2.—INTRAMOLECULAR CYCLIC TRANSFORMATIONS OF METHYL BIS (B CHLOROETHYL) AMINE

The distribution of radioactive sulfur mustard given intravenously to rabbits was studied by Bournsnel et al <sup>30</sup> The concentration of sulfur mustard in the plasma fell rapidly within a period of four hours while the concentration within the red cell layer remained relatively constant Seven per cent of the injected sulfur mustard was excreted into the bile in twenty minutes and 50 per cent was excreted within one hour The amount of radioactive sulfur fixed to the bone-marrow appeared to be of lesser magnitude than that fixed to other organs However, the quantity per gram of total nitrogen was of the same order

Friedenwald and Buschke<sup>31</sup> studied the effect of the nitrogen mustards upon corneal epithelium Cells which were exposed during the active phase of mitosis were unaffected by moderate concentrations of the chemical and went on to complete their division With continued exposure, however, all mitotic figures eventually disappeared The resting stage of the mitotic cycle was the most sensitive period Higher concentrations produced fragmentation of nuclei and abnormal chromosomal patterns which were transmissible through succeeding generations

Various histologic effects of the nitrogen mustards in experimental animals will be discussed below as related to similar effects noted in cases to be reported

## REVIEW OF THE LITERATURE

The dramatic though short-lived effects of the nitrogen mustards in the therapy of Hodgkin's disease were noted early in the course of clinical investigations with the chemical. Jacobson<sup>5</sup> reported the occurrence of remissions in 94 per cent of 120 courses administered to 29 cases. There were 8 failures, 3 of which were terminal and 2 radioresistant. One case had a temporary remission of fever for only three weeks. Four radioresistant cases responded well. Remissions lasted up to ten months.

Craver<sup>11</sup> reported 43 cases of Hodgkin's disease treated at the Memorial Hospital in New York. Constitutional symptoms responded favorably. Partial regression of lymph nodes, liver and spleen followed therapy. Pruritus and bone lesions responded poorly.

Wintrobe and Huguley<sup>10</sup> obtained good improvement in 17 and fair improvement in 5 of 32 treated cases. Fever responded dramatically in almost all cases. Improved well-being and appetite were noted in most cases. Remissions lasted from one to twenty-six months. The average duration of remissions was three months.

Zanes et al.<sup>12</sup> noted that remissions occurred in three types of patients with Hodgkin's disease: (1) patients who were radiosensitive or who had had no previous therapy, (2) patients with severe constitutional symptomatology, (3) radioresistant cases. Patients in the last group were occasionally resensitized to x-ray after a course of nitrogen mustard. Remissions averaged 2.8 months in length.

ApThomas and Collumbine<sup>13</sup> reported 21 treated cases. All improved following their first course. Improvement was noted in 12 of 13 cases who received a second course, and in 2 of 4 cases who received a third course of nitrogen mustard. The response was usually more rapid than previously noted with roentgen therapy. Remissions lasted from two to six months.

Alpert and Peterson<sup>14</sup> reported 8 previously untreated cases, 6 of whom had complete or partial remissions lasting three weeks to four months following HN<sub>2</sub> therapy. Heightened responses were obtained by the co-administration of x-ray therapy.

Talbott<sup>16</sup> obtained no response in 2 of 10 treated cases of Hodgkin's disease. Hettig<sup>16</sup> reported excellent remissions in 2, a partial remission in 1, and slight or no remission in 3 of 6 treated cases. Wilkinson and Fletcher<sup>18</sup> obtained satisfactory remissions in 3 of 4 previously untreated cases lasting up to 17 weeks. Sherry<sup>17</sup> reported remissions lasting from 44 days to 11 months in six cases of Hodgkin's disease.

Taffel<sup>19</sup> reported partial remissions in six cases lasting up to six months.

## MATERIALS AND METHODS

Methyl bis (B chloroethyl) amine\* (HN<sub>2</sub>) was used in the treatment of 50 cases of Hodgkin's disease at the J. H. Pratt Diagnostic Hospital, Boston Dispensary and West Roxbury Veterans Hospital. The diagnosis of Hodgkin's disease was made in almost every instance by biopsy of a suitable enlarged pe-

\* Methyl Bis (B chloroethyl) amine was supplied in generous amounts by the Merck Chemical Company through the cooperation of the National Research Council.

peripheral lymph node. In 2 cases with intraspinal involvement the diagnosis was made in the course of laminectomy and examination of excised tissue. No attempt was made in the analysis of this series of cases to differentiate sharply between various types of disease. We recognize that the growth potentiality of Hodgkin's disease, as of all neoplastic disease, varies considerably from case to case and sometimes in the same case. Our results in the most rapidly growing form of the disease known as Hodgkin's sarcoma were often as striking as with the least malignant types. With study of a larger series of cases in the future it may be possible to analyze more accurately the results of treatment in relation to the histologic picture. In any event the histologic picture of removed tissue was characteristic of the condition known as Hodgkin's disease and showed the histologic features of reticulum cell hyperplasia, increased reticulum, the presence of Reed-Sternberg giant cells and a variable degree of necrosis, eosinophilia and polymorphonuclear infiltration. The cases reported in this paper represent patients consecutively treated between December 1943 and December 1947. There were 29 males and 21 females. Fifteen of the 29 males were treated at the West Roxbury Veterans Hospital. The ages of the patients ranged from 19 to 62 years with a majority of cases below the age of 35. Initially the administration of nitrogen mustard was restricted to radioresistant or terminal cases. In 1946-1947, however, its use was extended to a few radiosensitive and previously untreated cases.

The chemical was packaged in 20 cc ampules, each containing 10 mg. Initially this was dissolved in 10 cc of saline and the required dose injected directly into the vein. Because of the frequent occurrence

TABLE 1—*Immediate Reactions Following 289 Doses of HN<sub>2</sub>*

	per cent
Nausea and vomiting	93.2
Chills	12.4
Fever	6.8
Headache	1.7
Thrombosis	1.0
Cyanosis	0.7
Dyspnea	0.7
Diarrhea	0.3
No reaction	6.8

of venous thromboses it became our practice early to inject the material into the rubber tubing of a freely flowing saline infusion. A course of therapy consisted of four to six injections of nitrogen mustard administered on successive or alternate days. An initial dose of 4 to 5 mg. was given on the first day. If this amount was well tolerated, succeeding doses were increased in 1 mg. amounts.

On each visit the presenting symptoms were recorded, the patient examined, and the blood counts obtained. These usually included white blood counts, hemoglobin and reticulocyte levels, platelet counts and a differential count of the white cell cells. Hemoglobin determinations were made with the Cenco hemoglobinometer. Reticulocyte and platelet counts were performed by the method of Dameshek.<sup>22</sup> Stereal bone-marrow punctures were performed prior to therapy in most cases and whenever possible at various intervals following HN<sub>2</sub> administration. The spinous process was often utilized for marrow aspirations in cases studied serially. Serial lymph node aspirations were performed whenever feasible.

## RESULTS

### *Immediate Reactions*

Table 1 lists the immediate reactions following the use of 289 doses of HN<sub>2</sub>. Nausea and vomiting occurred in 93.2 per cent of all cases. This usually began one to three hours after the injection and lasted for two to four hours. The cause of the nausea and vomiting has not been elucidated. It has been attributed to central medullary stimulation and to hemorrhage and necrosis of the gastrointestinal

tract. The marked excretion of the chemical into the bile<sup>30</sup> and subsequently into the second portion of the duodenum may, by causing irritation, be an important factor in the regularity of the occurrence of nausea and vomiting. However, Karnofsky<sup>9</sup> et al. found that the gastrointestinal lesions occurred even when the bile duct was clamped.

There were no reactions in 6.8 per cent of cases. Four of this group responded well making it unlikely that the injected material was inactive. Various attempts were made to reduce the severity of the nausea and vomiting by the co-administration of pyridoxine, morphine and barbiturates.\* Pyridoxine was discontinued because of its possible inactivation by nitrogen mustard.<sup>7</sup> Barbiturates had little value. Morphine appeared to allay much of the apprehension incident to the severe nausea and vomiting. It has been our practice to administer one-eighth grain of morphine sulphate subcutaneously in all hospital cases just before HN<sub>2</sub> administration. Shaking chills were observed in 12.4 per cent of cases. These usually occurred one-half to one hour after HN<sub>2</sub> administration and prior to the onset of nausea and vomiting. Chills recurred with successive doses in 7 cases. Morphine tended to diminish such recurrences. Fever either followed the chills or occurred independently in 6.8 per cent of cases. The exact cause for the pyrogenic reactions is unclear. In rabbits, Boursnel, et al.<sup>36</sup> demonstrated an alteration of serum proteins by mustard gas. These proteins possess different immunologic properties. The presence of such foreign proteins may be etiologic in the occurrence of chills and fever.

Headache was a prominent complaint in two patients who had developed a striking aversion to nitrogen mustard. Dyspnea and cyanosis occurred rarely and generally responded well to sedation.

When HN<sub>2</sub> was injected directly into the vein, thrombosis occurred commonly. The incidence of thrombosis disappeared almost completely with the administration of the chemical into the rubber tubing of a rapidly flowing infusion. Two patients who received tris (B-chloroethyl) amine developed thromboses of all injected veins, even when the material was injected into the rubber tubing.

In 4 cases, HN<sub>2</sub> was administered prophylactically in the form of weekly and biweekly injections in the attempt to maintain a remission induced by a course of medication. The reactions were of such severity that this form of therapy had to be discontinued. The same patients, when treated during an active phase of their disease had much milder reactions. It is possible that the actively proliferating granulomatous tissue present in relapse may selectively absorb the nitrogen mustard. During periods of remissions, however, large quantities of unabsorbed chemical may be available for the production of side reactions.

#### *Type and Duration of Response*

During the first three years of these studies only terminal or radioresistant cases were subjected to therapeutic trial. The results in this group are not as favorable

\* More recently a solution of procaine has been given intravenously immediately following HN administration.

as those obtained in less advanced cases treated during the past year. For purposes of analysis, all cases are however grouped together.

Figure 3 shows the type of response obtained in the first fifty cases of Hodgkin's disease treated with 102 courses of  $\text{HN}_2$ . In 79.4 per cent a complete or partial response to therapy occurred. In 20.6 per cent, there was no response.

The duration of the response ranged from 17 to 331 days\* (figure 4). Remissions lasting less than fifty days were noted in 41.7 per cent, 35.2 per cent developed good responses lasting from 50 to 331 days.

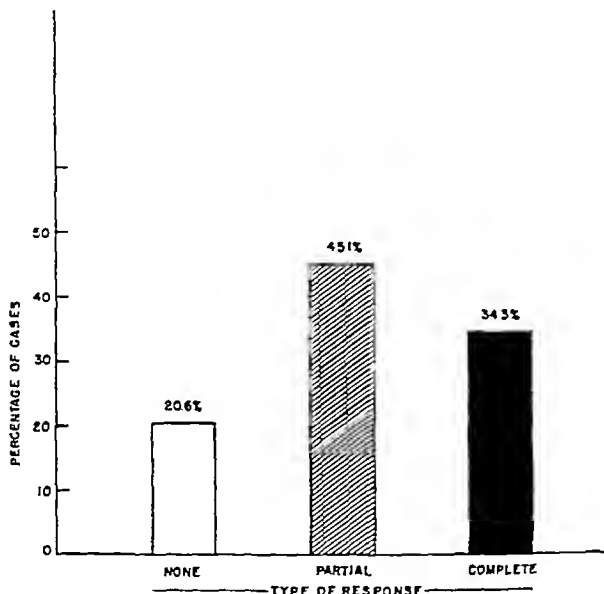


FIG. 3.—TYPE OF RESPONSE OBTAINED FOLLOWING 102 COURSES OF  $\text{HN}_2$  IN 50 CASES OF HODGKIN'S DISEASE

Twenty-three patients received a single course of  $\text{HN}_2$ , 11 patients received 2 courses, 9, 3 courses, 4, 4 courses, 2, 5 courses, and 1, 8 courses of  $\text{HN}_2$ . The general results obtained with successive courses of  $\text{HN}_2$  are roughly comparable to the composite results for all courses, with the same proportion of successes and failures. The duration of the response obtained in sixteen patients who received multiple courses with varying dosage schedules was approximately proportional to the total dose administered.

Thirty-one patients in this series were regarded as having become resistant to x-ray therapy and in 13 of these, all of whom appeared to be running a progressively downhill course, good remissions following therapy were obtained. Some of the most spectacular results were seen in cases that were virtually moribund on

\* The results as reported in this paper are based on findings ending December 15, 1947.

admission (cases 1, 23 and 28) There can be no doubt that many patients of this group have had a moderate prolongation of their life span, as well as a more comfortable existence after having become completely resistant to further x-ray therapy Nitrogen mustard was particularly useful in 5 cases with severe x-ray dermatitis

Nine patients failed to show any response following the initial and subsequent courses of nitrogen mustard therapy

In 7 patients, roentgen therapy was given just before the administration of nitrogen mustard In 4 of this group (cases 10, 21, 28 and 40), there was definite prolongation of the length of the remission In 8 cases, x-ray therapy was given

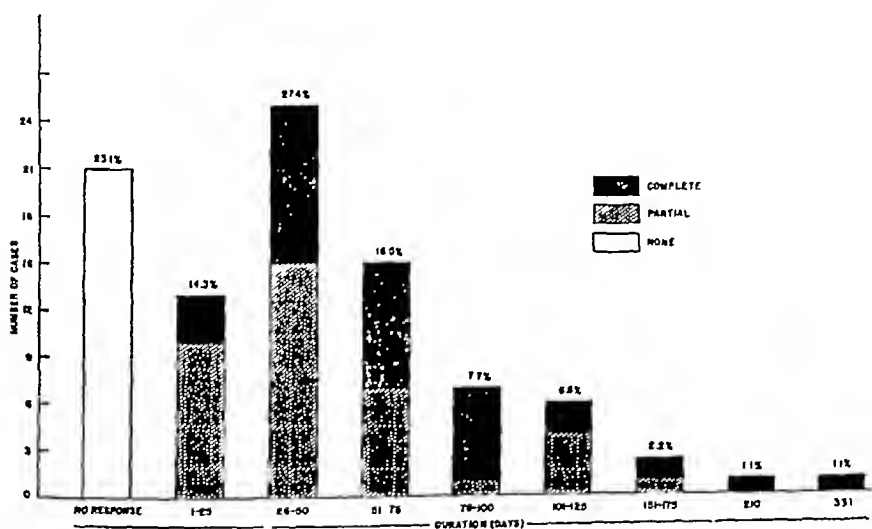


FIG 4—DURATION OF RESPONSES FOLLOWING 91 COURSES OF  $\text{HN}_2$  IN 50 CASES OF HODGKIN'S DISEASE

following the administration of nitrogen mustard In 3 of these cases residual lymphoid organs regressed with unusual rapidity when x-ray therapy was given

Four cases received  $\text{HN}_2$  as their sole initial therapy The remissions lasted from 36 to 120 days in 3 cases Case 9 received three courses of nitrogen mustard which resulted in partial remissions lasting 36, 30 and 51 days respectively The latter course had been combined with roentgen therapy Case 49, who developed a severe hemorrhagic complication due to thrombocytopenia has nevertheless had an excellent remission which had continued to the time of this writing

Case 31, received combined  $\text{HN}_2$ -roentgen therapy following which he had a complete remission lasting 210 days

The number of patients given  $\text{HN}_2$  as the first therapeutic procedure is too small to permit statistical evaluation It appears, however, that the remissions obtained are of much shorter duration than is usually the case following roentgen therapy

Similar results were reported by Alpert and Peterson<sup>14</sup> The remissions appear to be definitely longer if combined  $\text{HN}_2$  and x-ray therapy is administered



## EFFECTS OF NITROGEN MUSTARD THERAPY ON CLINICAL MANIFESTATIONS

*Systemic Manifestations*

A majority of the patients in this series had the characteristic constitutional symptomatology of severe, long standing Hodgkin's disease, i.e., malaise, case

TABLE 2.—*Response of Signs and Symptoms Following HN<sub>2</sub> Therapy (50 cases of Hodgkin's Disease)*

Signs and Symptoms	Number of Adminis- trations	Percentage Completely Relieved	Percentage Partially relieved	Percentage Unrelieved
<b>A. Constitutional symptoms</b>				
Fatigability	74	59.5	22.9	17.6
Anorexia	62	77.4	9.7	12.9
Fever	46	58.7	4.3	37.0
Sweats	25	84.0		16.0
Pruritus	15	40.0	33.3	26.7
Chills	5	80.0		20.0
<b>B. Lymphoid involvements</b>				
Adenopathy	84	38.1	32.1	29.8
Splenomegaly	46	39.1	32.6	28.3
Edema	14	14.3	50.0	35.7
Gastro-intestinal complaints	6	83.3		16.7
<b>C. Mediastinal involvement</b>				
X ray changes	31	29.0	38.7	32.3
Cough	20	30.0	20.0	50.0
Dyspnea	17	17.6	35.3	47.1
Hoarseness	7			100.0
Dysphagia	3			100.0
Superior vena caval syndrome	2	100.0		
<b>D. Hepatic involvement</b>				
Hepatomegaly	30	26.6	10.0	63.4
Jaundice	4	50.0		50.0
Ascites	3		66.6	33.4
<b>E. Neurologic involvement</b>				
1. Intraspinal				
Paraplegia	4		50.0	50.0
Back pain	7	71.4	28.6	
? incontinence	3		33.3	66.7
2. Perispheral				
Paralysis upper extremity	3			100.0
Pain—back	9	100.0		
—shoulder	4	25.0	25.0	50.0
Horner's syndrome	11	9.1	9.1	81.8
<b>F. Osseous involvement</b>				
	21			100.0

of fatigability, anorexia, fever, night sweats, pruritus and chills. Fatigability and anorexia were relieved (completely or partially) in 82.4 and 87.1 per cent of cases respectively (table 2.)

Following a course of nitrogen mustard therapy and after the immediate reaction had subsided, there usually occurred a marked upsurge in vitality and well-

being Those patients who had previously received roentgen therapy usually commented upon the greater subjective improvement which followed the administration of nitrogen mustard

Seven patients were treated with nitrogen mustard shortly after the recognition of their disease In 2 cases (21 and 40) this was administered after a partially effective or ineffectual course of roentgen therapy Single courses of  $\text{HN}_2$  resulted in excellent remissions lasting respectively 331 and 169 days and continuing to the time of this writing Case 21 illustrates a striking response in constitutional symptoms and a prolonged remission

#### CASE 21 (FIGURE 5)

*P D C* A 36 year old white male began to notice easy fatigability weakness and marked weight loss in 1944 In January 1946 weakness and fatigability became much more pronounced In September 1946 he was found to have continuous fever A gnawing sensation in the mid abdomen was relieved by food and medication He was admitted in October 1946 to the West Roxbury Veterans Hospital

*Physical Examination* Temperature 99.4 F The patient was a well developed rather well nourished white male His voice was hoarse The eyes were slightly protuberant The right lobe of the thyroid was more readily palpable than the left The chest was clear and resonant throughout A grade II systolic murmur was heard just to the left of the sternum and in the fourth interspace The heart was otherwise negative The liver and spleen were not felt There were bean sized axillary (right) lymph nodes

*Laboratory Data* Blood counts leukocytes 18 800 erythrocytes 3 600 000 hemoglobin 11.1 Gm differential polymorphonuclear neutrophils 80 per cent monocytes 5 per cent lymphocytes 15 per cent The urine was negative Blood sedimentation rate was 55 mm per hour, Mazzini test was negative Sputum was negative for tubercle bacilli Basal metabolic rate was plus 34.5 per cent A roentgenogram of the chest showed evidence of mediastinal lymphadenopathy Biopsy of an enlarged axillary node revealed the presence of Hodgkin's granuloma

*Course* The patient ran a febrile course with temperature elevations up to 101.4 F Beginning October 31 1946 the patient was given 18 roentgen treatments over the anterior and posterior chest and to the right axilla However he continued to run a low grade fever and to lose weight Several right axillary nodes were still palpable Repeat roentgenograms of the chest showed a complete reduction in the size of the right upper mediastinal mass A flat plate of the abdomen at this time revealed an enlarged spleen extending down to about two inches above the iliac crest On January 4 1947 a course of nitrogen mustard therapy was begun consisting of 4 5 6 and 7 mg doses administered on successive days Nausea and vomiting occurred two hours after each administration and lasted from one half hour to three hours The fever subsided promptly Shortly after the nitrogen mustard therapy there followed a reduction in the white blood count from 18 000 to 6600

The patient developed a marked improvement in his appetite and gained about 60 pounds in weight He had a remarkable upsurge in strength and sense of well being Lymphadenopathy disappeared entirely and the spleen regressed completely He was then observed at regular intervals in the outpatient department and continued in an excellent state of remission to the time of writing almost a year later

*Fever*, which was a presenting complaint in 30 cases, was completely relieved in 58.7 per cent following  $\text{HN}_2$  treatment (figure 6)  $\text{HN}_2$  appeared to be less effective in 13 terminal patients who showed the typical Pel-Ebstein type of relapsing fever Two of these cases had responded well to a previous course of therapy Cases 19 and 24 had associated infections, i.e., a chronically draining bronchopleural fistula, and an ascending urinary tract infection respectively The infections were not affected by the  $\text{HN}_2$  treatment

Severe night sweats were relieved in 84 per cent of cases Of 4 cases, in which night

sweats did not respond to  $\text{HN}_2$  therapy, 3 were terminal, and case 19 noted above had a chronic infectious process

Pruritus was present in 10 cases prior to nitrogen mustard therapy. Improvement followed in 73.3 per cent. The pruritus was of such intensity in case 38 that the patient forcefully removed all toenails and produced deep excoriations of the skin.

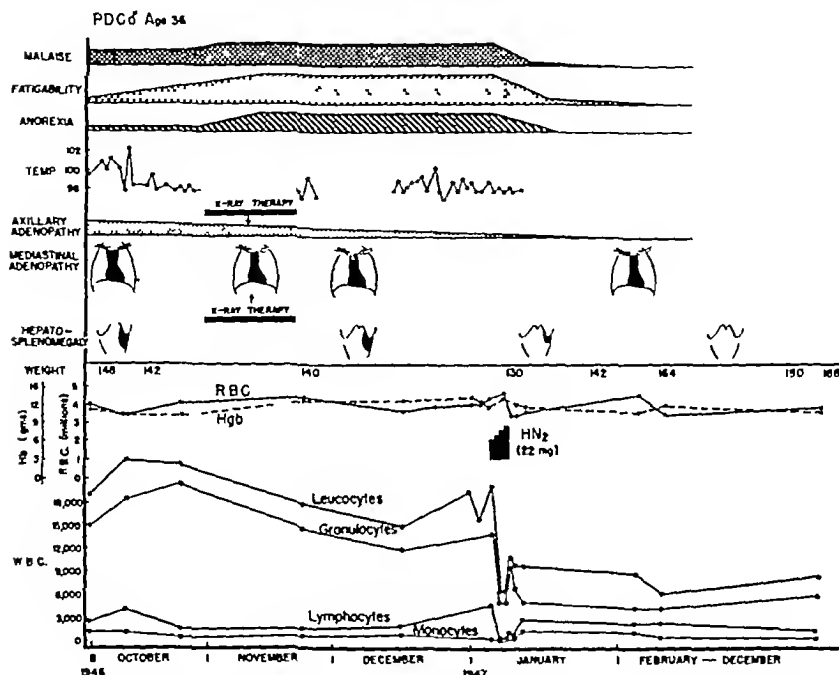


FIG. 5.—EFFECTS OF  $\text{HN}_2$  IN PATIENT (CASE 21) WHO HAD DEVELOPED RESISTANCE TO X-RAY THERAPY. The constitutional symptoms fever, mediastinal adenopathy, generalized lymphadenopathy, and splenomegaly were promptly relieved following  $\text{HN}_2$  therapy.

Roentgen therapy could no longer be administered because of severe x-ray dermatitis. Considerable relief and healing of the excoriations followed each of two courses. Four terminal cases showed no response.

Chills were present in 5 patients, 4 of whom responded well to therapy. In one case there was a progressively downhill course.

### Lymphoid Involvement

**Lymphadenopathy.** Regression of enlarged glands occurred in 70.2 per cent of the cases. In a few patients this was noted as early as twelve hours after the injection of the first dose of nitrogen mustard. Rarely, slight initial enlargement preceded subsequent regression of glands. Twenty-eight of the 44 patients with lymphadenopathy were referred to us for nitrogen mustard therapy because of their radio-

resistant state, of this group, 60.3 per cent showed a complete or partial response. The following case is described in detail to illustrate the response obtained in a patient with a fluctuant supraclavicular mass and a superimposed severe radio-dermatitis.

## CASE 39

M V, a 25 year old white female noted the onset of left supraclavicular adenopathy in December 1945. A biopsy taken in March 1946 revealed the presence of Hodgkin's disease. Roentgen therapy was

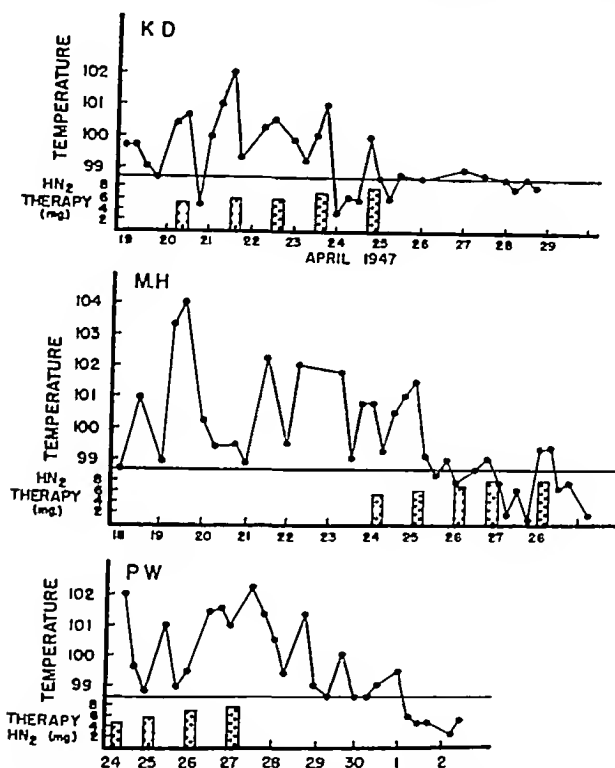


FIG. 6—EFFECT OF HN<sub>2</sub> IN REDUCING FEVER IN 3 CASES OF SEVERE HODGKIN'S DISEASE

administered in the left supraclavicular axillary and mediastinal areas with gradual improvement. Adenopathy recurred in October 1946 and continued to increase despite intensive roentgen therapy. The left supraclavicular mass became fluctuant and soon broke through the superimposed skin which showed evidence of a severe radiodermatitis. The roentgenologist referred her for nitrogen mustard therapy in May 1947.

**Physical Examination.** The patient showed pallor. She presented a large hard but superficially fluctuant left supraclavicular mass 12 cm in diameter superimposed by a deeply pigmented area of skin (fig. 7a). There was a number of smaller cervical, right supraclavicular and left axillary glands. The spleen and liver were not enlarged.

**Laboratory Data.** Blood counts: Leukocytes 20,050; erythrocytes 3,630,000; hemoglobin 11.0 Gm; reticulocytes 0.9 per cent; platelets 785,500; differential count: polymorphonuclear neutrophils 57

per cent, band forms 17 per cent monocytes 8 per cent lymphocytes, 13 per cent Bone marrow differential polymorphonuclear neutrophils, 25 6 per cent band forms 14 2 per cent, metamyelocytes, 19 2 per cent, myelocytes 15 8 per cent, promyelocytes 1 2 per cent myeloblasts, 0 5 per cent reticulum cells 0 2 per cent plasma cells 2 0 per cent megakaryocytes plentiful erythrocyte granulocyte ratio 1 7 The urine was negative The Hinton test was negative The blood sedimentation rate was 72 mm per hour Roentgenograms of the chest showed left supraclavicular and mediastinal masses

*Course* The left supraclavicular mass was incised and drained The patient was then started on a course of HN consisting of 5, 5 6, 7 and 8 mg administered on successive days Each dose was followed by rather severe nausea and vomiting The wound healed rapidly and all glandular adenopathy subsided completely leaving only a small area of induration in the left supraclavicular region (fig 7b) A repeat roentgenogram of the chest showed reduction in the size of the mediastinal and supraclavicular masses The remission lasted approximately three months when supraclavicular and axillary adenopathy recurred The patient received a second course of HN consisting of 5 6 7 and 8 mg administered on alternate days There was again regression of all the glands

Eight cases failed to show any regression of lymph nodes following HN<sub>2</sub> therapy Case 5 had had two previously successful remissions following HN<sub>2</sub> and lasting 74 and 56 days respectively Four patients showed no response to the initial course of the therapy

Three patients with HN<sub>2</sub> resistant glandular enlargements responded unusually well to roentgen therapy given shortly after administration of one course of HN therapy Case 9 is described in detail, and case 14, is presented briefly

#### CASE 9

*V K* a 25 year old white female was first seen in September 1946 She presented a six month history of anorexia weakness fatigability weight loss fever and cervical adenopathy Biopsy of an enlarged gland revealed Hodgkin's disease probably of the sarcoma type

*Physical Examination* The patient showed moderate pallor and marked weight loss She had a right Horner's syndrome There was generalized adenopathy Supracardiac dullness was 8 cms in diameter The spleen was three finger's breadth below the left costal margin

*Laboratory Data* Leukocytes 10 800 erythrocytes 3 810 000 hemoglobin 7 6 Gm reticulocytes 0 5 per cent platelets 663 940 differential polymorphonuclear neutrophils 79 per cent band forms, 10 per cent monocytes 4 per cent lymphocytes, 7 per cent Bone marrow differential polymorphonuclear neutrophils 21 5 per cent band forms 29 5 per cent metamyelocytes 21 0 per cent myelocytes 10 5 per cent promyelocytes 0 5 per cent myeloblasts 0 5 per cent plasma cells 0 3 per cent reticulum cells 0 3 per cent reticulum cells 2 0 per cent megakaryocytes plentiful, erythrocyte granulocyte ratio 1/3

The urine was negative The Hinton test was negative Roentgenograms of the chest showed marked mediastinal widening (fig 8a)

*Course* The patient received 4 mg of HN on September 25 1946 Three-quarters of an hour later she became moderately dyspneic and cyanotic This responded gradually to sedation She received two additional doses consisting of 4 and 6 mg on September 29 and 30 respectively These were followed by the usual reactions of nausea and vomiting A roentgenogram of the chest taken five days after the completion of nitrogen mustard therapy showed marked regression of the mediastinal mass Further reduction was noted seven days later (fig 8b) There was complete regression of all peripheral glands

The patient had a remission which lasted 36 days Following this she developed recurrent cervical adenopathy A second course of HN consisting of 4 5 5 6 and 6 mg was administered on alternate days beginning November 4 1946 Enlarged glands regressed completely Thirty three days later the patient noted the onset of anorexia weight loss left cervical and bilateral axillary glands and a walnut sized parasternal mass in the second interspace on the right The spleen descended one finger's breadth below the left costal margin A third course of HN consisting of 4 5 6 and 7 mg was administered on alternate days beginning January 6 1946 A chill followed the second third and fourth doses within

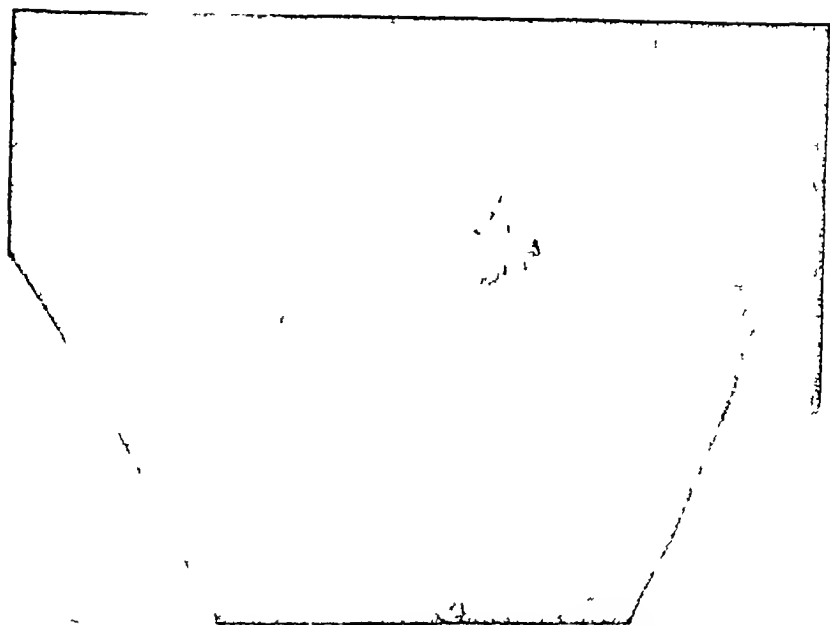


FIG 7—RESPONSE OF RADIORESISTANT SUPRACLAVICULAR GLAND WITH SUPERIMPOSED BROKEN DOWN  
PIGMENTED SKIN EXUDING SERO-PURULENT MATTER (CASE 39)

(a) PRIOR TO  $\text{HN}_2$  THERAPY

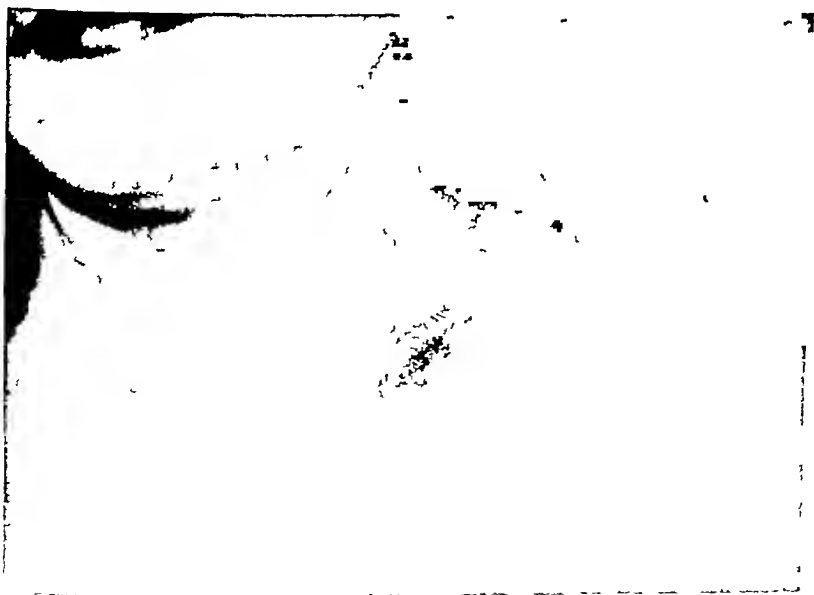


FIG 7—(b) AFTER COMPLETION OF COURSE OF  $\text{HN}_2$  THERAPY



FIG 8—RESPONSE OF MEDIASTINAL ADENOPATHY FOLLOWING  $\text{HN}_2$  THERAPY (CASE 9)  
(a) PRIOR TO FIRST COURSE OF THERAPY

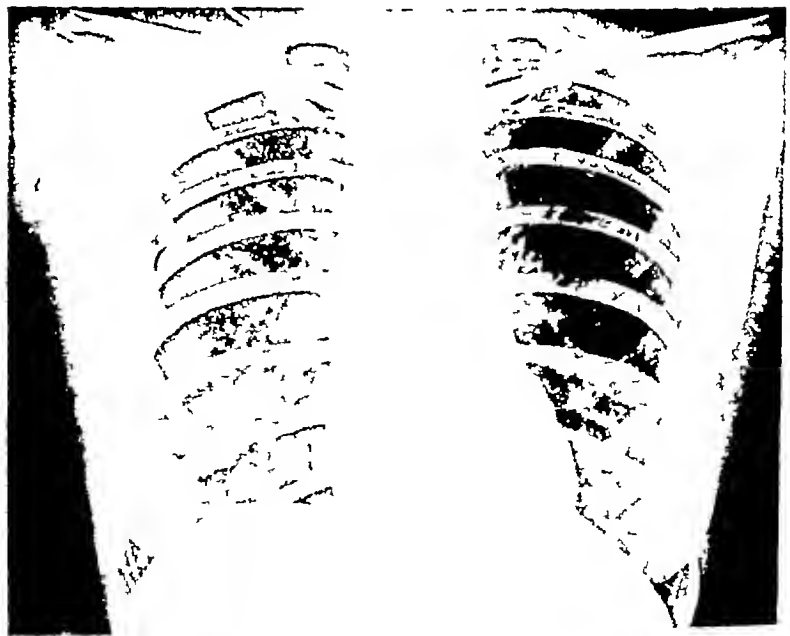


FIG 8—(b) SAME PATIENTS TWELVE DAYS AFTER COMPLETION OF FIRST COURSE OF THERAPY

one half three-quarters and two hours respectively. The usual reactions of nausea and vomiting followed each dose. The patient had a marked upsurge in well being and gained five pounds in weight. Glandular adenopathies regressed approximately 25 per cent. Roentgen therapy was then administered to the parasternal and left supraclavicular areas (1200 r each). Rapid resolution of residual glands occurred. This remission lasted sixty-one days. Further nitrogen mustard was refused.

#### CASE 14

This patient a long standing example of Hodgkin's disease with the presenting complaint of severe constipation showed a very large mass in the left lower quadrant of the abdomen dipping into the pelvis. A course of nitrogen mustard therapy was completely ineffectual. Roentgen therapy was then given to both the left lower quadrant (600 r) and the right lower quadrant (400 r) and was followed by rapid resolution of the pelvic masses and a dramatic relief of constipation.

*Splenomegaly* Seventy-one and seven-tenths per cent of the cases with splenomegaly showed complete or partial regression of the enlarged spleen following  $\text{HN}_2$  therapy. This corresponds roughly to the results obtained with lymphadenopathies. The striking affinity of nitrogen mustard for the various lymphoid organs was noted by Pappenheimer and Vance<sup>3</sup> and by Graef et al.<sup>17</sup> Atrophy of lymph nodes, spleen and thymus has been demonstrated in normal mice, rats, rabbits, dogs, chickens and pigeons following the administration of nitrogen mustard.

*Edema* Edema due to pressure by enlarged lymph nodes or to lymphatic obstruction was present in 12 cases prior to the initiation of nitrogen mustard therapy. Two patients had edema of the lateral half of the breast, secondary to enlarged axillary nodes. Case 12 presented an orange-sized right axillary mass with edema of the lateral half of the right breast. A partial response followed the administration of 26 mg. of  $\text{HN}_2$ . Roentgen therapy then brought about rapid and complete regression of the axillary glands as well as edema. Case 2 had very large axillary masses and edema of both breasts. A partial remission lasting one month was induced by the first course of nitrogen mustard. Two subsequent courses, however, were without effect.

Gross edema of the lower extremities was present in four patients.

#### CASE 30

*W J O*, a 44 year old white male was first seen in March 1947. Six years previously he had noted the presence of a large mass in the left inguinal region. A biopsy revealed Hodgkin's disease. Intensive roentgen therapy induced a complete regression. Further roentgen therapy was administered as needed with recurrent glandular adenopathies. Increasing radioresistance was noted. Six months prior to admission the patient developed massive edema of the left lower extremity extending to the lumbar area. Roentgen therapy was initially effective in reducing the edema. Three months prior to admission the patient developed extreme edema of the right lower extremity and scrotum. Roentgen therapy was ineffectual.

*Physical Examination* The patient had massive edema amounting to elephantiasis of both lower extremities and the scrotum. There was an x-ray dermatitis of the left inguinal, gluteal and lumbar areas. The liver was felt four fingers breadth below the right costal margin and the spleen three fingers breadth below the left costal margin.

*Course* The patient received three daily injections of  $\text{HN}_2$ . Within ten days the edema had completely subsided. The patient remained well for three weeks when he suddenly developed a severe pain in the left groin radiating to the hip and small of back. The edema of the left lower extremity recurred. Three days later, the patient had a sudden massive gastrointestinal hemorrhage. He rapidly lapsed into shock and died twelve hours later.



*Postmortem* At autopsy there was a fistulous communication between the p.l.v.s the retroperitoneal lymph nodes and rectosigmoid. The descending colon contained much freshly coagulated blood. Both ureters were compressed by a large retroperitoneal mass producing bilateral hydronephroses and hydrocephroses. There were large nodes in both inguinal regions with compression of the femoral artery and vein on the left.

In another patient (Case 23), massive ascites and marked edema of both lower extremities was present. The energetic use of paracentesis, transfusions, plasma, albumin and  $\text{HN}_2$  therapy brought about a marked reduction of the edema and ascites and a well-defined remission.

Edema of the upper extremities was present in 2 cases. Case 8, with scar tissue in the left supraclavicular and axillary nodes, was treated with  $\text{HN}_2$ , which brought about an approximately 30 per cent reduction in the edema. Two subsequent courses were however, completely ineffectual.

Three patients had edema suggesting superior vena caval obstruction. These will be discussed below, under mediastinal involvement.

As noted above, edema may be due to lymphatic or venous obstruction, pressure from enlarged glands, or by scar tissue. When due to enlarged glands, nitrogen mustard was found to be moderately effective. When due to scar tissue little or no effect was obtained. It is probable that  $\text{HN}_2$  is far less productive of scar tissue than is roentgen therapy.

### *Mediastinal Involvement*

*Roentgen Changes* X-rays of the chest were performed routinely in all cases. Twenty-one cases showed radiologic evidence of pulmonary or mediastinal involvement. In 7, there was no associated symptomatology. The response of such asymptomatic mediastinal adenopathy to nitrogen mustard therapy is shown in figure 8.

Twelve patients had symptoms referable to their pulmonary pathology, i.e., cough, dyspnea, hoarseness and dysphagia. This group usually had extensive mediastinal involvement. Eleven cases had been previously declared radioresistant. The response to the  $\text{HN}_2$  therapy was only moderately effective in this group. The following case illustrates a partial response to  $\text{HN}_2$  therapy and a better response to combined  $\text{HN}_2$  and roentgen therapy.

#### CASE 25 (FIGURE 9)

*K. D.*, a 37 year old white housewife was the first seen on February 3, 1947. Five and one half years ago she noted the onset of right cervical adenopathy and upon roentgenographic examination of the chest was shown to have a mediastinal mass. A biopsy revealed the presence of Hodgkin's disease. Roentgen therapy was then administered to the cervical and mediastinal areas with prompt improvement. During the following three years the patient received five courses of roentgen therapy each of which induced a short remission. In March 1947 an episode of severe cough, chills, fever (103 F), dyspnea and fatigability developed.

*Physical Examination.* The patient had a marked radio-dermatitis over the anterior and posterior chest. No adenopathy could be made out. Supracardiac dullness was 17 cm. in diameter. Bronchial breath sounds were present over the left apex. The liver and spleen were not palpable.

*Laboratory Data* Blood counts: leukocytes 8,500, erythrocytes 4,170,000, hemoglobin 12.1 Gm, reticulocytes 1.6 per cent, platelets 638,880, differential polymorphonuclear neutrophils 65 per cent, band forms 12 per cent, eosinophiles 2 per cent, basophiles 1 per cent, monocytes 6 per cent, lympho-

cytes, 14 per cent. The urine was negative. The Hinton test was negative. Roentgenograms of the chest showed extensive anterior mediastinal enlargement (fig. 9) and bilateral infiltration of the lung.

*Course.* The patient was started on a course of HN consisting of 4, 5, 6, and 7 mg administered on alternate days beginning February 3, 1947. Shaking chills occurred two hours after the first, second and fourth injections. The usual reactions of nausea and vomiting followed. The patient experienced a marked reduction of cough and dyspnea as well as a striking improvement in vitality. The remission lasted for about three weeks when in March 1947 she had another episode of fever (104 F) cough and dyspnea. A similar episode occurred one month later. A roentgenogram of the chest revealed a large mediastinal mass occupying almost the entire upper chest. A second course of HN<sub>2</sub> was instituted on April 20, 1947 and consisted of daily injections of 6, 6, 6, 7 and 8 mg. The usual reactions of nausea and vomiting occurred. The fever subsided gradually. Roentgen therapy to the mediastinum in a total dosage of 1200

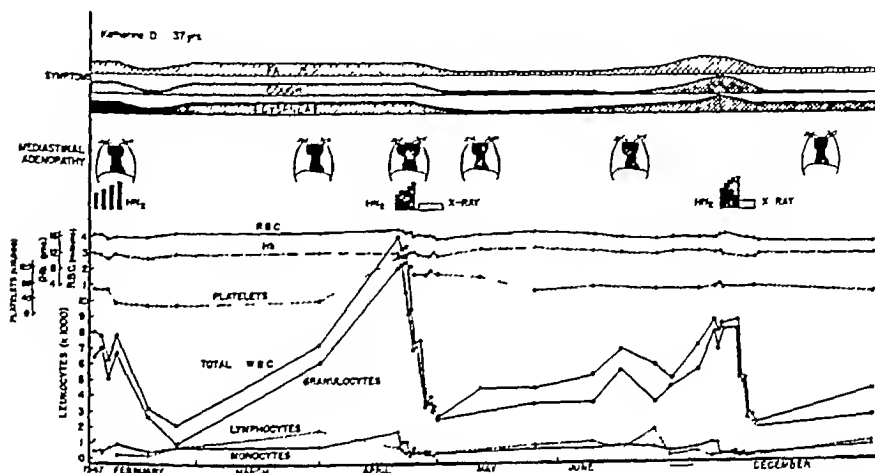


FIG. 9—EFFECTS OF NITROGEN MUSTARD IN PATIENT WITH MASSIVE MEDIASTINAL INVOLVEMENT WHO HAD BECOME MARKEDLY RADIORESISTANT (CASE 25). A distinct, although temporary response took place with the use of HN<sub>2</sub> but best effects were obtained when HN<sub>2</sub> was given first and then followed by x-ray therapy.

r was then given. A marked improvement again occurred with considerable relief of cough, dyspnea and fatigability. Roentgenograms of the chest showed marked regression in the size of the mediastinal mass. A third course of HN<sub>2</sub> was instituted on September 30, 1947 and consisted of daily injections of 5, 6, 7 and 8 mg. Roentgen therapy was also given through axillary portals: 450 r to the right axilla and 525 r to the left axilla. The patient showed an improvement in well-being but no further change in the size of the mediastinal mass was noted.

There can be no question that combined HN<sub>2</sub> and x-ray therapy was productive in this patient of more prolonged and effective remissions than HN<sub>2</sub> alone. A symptomatic response occurred following the third course although no further roentgenographic change could be noted.

*Cough.* Cough was a presenting symptom in 13 cases. Complete or partial improvement followed nitrogen mustard therapy in 50 per cent of the cases. In the other half, no response occurred and the patients ran a progressively downhill course.

*Dyspnea.* Dyspnea was notable in 15 cases prior to therapy. Cases 18, 42, and 43

showed no mediastinal involvement but had extensive and generalized involvement with Hodgkin's disease. Case 23 had massive ascites. Cases 22 and 50 had a massive hydrothorax as well as ascites. The results obtained within this group were generally unsatisfactory (table 2).

*Hoarseness and Dysphagia* All cases displaying these symptoms represented far advanced radioresistant cases. No improvement was noted following  $\text{HN}_2$  therapy.

*Superior Vena Cava Syndrome* Two patients showed signs of superior vena cava obstruction. Case 11 developed puffiness of the eyelids, suffusion of the conjunctivae, and swelling of the cheek during his course of  $\text{HN}_2$ . This gradually subsided as did the hilar adenopathy. Case 1 is described in detail (previously reported).<sup>9</sup>

#### CASE 1

*L W*, a 33 year old housewife, was first seen in 1941 because of axillary adenopathy. Her father had died of Hodgkin's disease and her mother of polycythemia vera. A biopsy revealed the presence of Hodgkin's disease. In rapid succession nodes appeared in the axillae, neck, and mediastinum. Roentgen therapy was given with excellent results initially; subsequent results were poor. In the summer of 1942, dyspnea and cough developed and a thoracentesis was required for the pleural effusion. Complete motor and sensory paralysis of the right arm appeared in the spring of 1943 and the limb gradually increased threefold in size. Cough, weakness, and dyspnea became worse. Lymph node masses increased and in the fall of 1943 the patient was bed ridden and failed to respond to further x-ray treatment.

*Physical Examination.* The patient was extremely ill and very cyanotic with a shallow dry cough and gasping respirations. The face and neck were greatly swollen and distorted and there was pitting edema over the upper thorax. The breasts were large and edematous. The right arm was greatly swollen and completely paralyzed. The left side of the neck bulged with a hard, irregular mass extending into the supraclavicular fossa. Both axillae were occupied by hard, irregular masses of nodes extending on the right side to the lower chest. The percussion note was dull to flat over both thoraces and the breath sounds were diminished. There was no enlargement of the spleen or liver and no inguinal adenopathy. The temperature ranged from 98 to 103 F and the pulse from 100 to 140 per minute.

*Laboratory Data.* Blood counts: leukocytes 8500, erythrocytes 3,910,000, hemoglobin 86 per cent, differential polymorphonuclear neutrophils 73 per cent, eosinophils 11 per cent, monocytes 13 per cent, lymphocytes 3 per cent. The Hinton test was negative. The urine was negative. The blood sedimentation rate was 40 mm per hour. Roentgen examination disclosed no mediastinal mass but decided infiltration of the lower two-thirds of both lung fields was present.

*Course.* On December 7, 1943, the patient was started on a course of tris (B-chloroethyl)amine administered on alternate days for four doses (11 mg per kilogram of body weight). This was injected by the direct syringe method. Improvement started after the second dose and continued over a period of two weeks. The patient felt much better; the fever and cyanosis disappeared; the dyspnea being improved and cough improved. The lymph node masses shrank 60 to 70 per cent; the breasts became smaller; the disfiguring edema of the face and neck receded entirely and the hugely swollen arm returned almost to normal size. Roentgenograms of the chest revealed no change in pulmonary infiltration.

The dramatic therapeutic remission persisted four weeks when it was interrupted by a sudden severe attack of pulmonary edema which quickly resulted in death. Postmortem examination was not obtained.

The results obtained in cases with extensive mediastinal involvement were on the whole not as satisfactory as those obtained with lesser degrees of mediastinal involvement. However, a more comfortable existence as well as a moderate prolongation of life was achieved with nitrogen mustard therapy. In patients who had been previously subjected to intensive roentgen therapy the resultant fibrosis within and around the mediastinal tumor mass may well occlude the vascular

avenues of approach. With little or no previous Roentgen therapy mediastinal tumors appeared to respond more satisfactorily.

### *Hepatic Involvement*

**Hepatomegaly** Hepatomegaly was present in 15 cases. Thirty-six and six-tenths per cent showed regression following  $\text{HN}_2$  therapy. Those who failed to respond were radioresistant terminal cases. Individuals with lesser degrees of hepatomegaly appeared to respond satisfactorily.

**Jaundice** In four cases hepatic enlargement was associated with jaundice. Two responded well while the other two showed no signs of improvement and in fact became worse following therapy. The following case is illustrative of a possible aggravation of liver dysfunction following  $\text{HN}_2$  therapy.

### CASE 35

**H. M.**, a 45 year old white male was first seen on April 16, 1947. Eight months prior to admission he noted the presence of a mass in the right cervical region which gradually increased in size until it filled the entire right side of the neck. He developed marked fatigability, night sweats and pruritus. A biopsy revealed the presence of Hodgkin's disease. Roentgen therapy produced a short remission. Subsequent roentgen therapy was completely ineffectual and the patient became progressively more disabled with severe night sweats and fever.

**Physical Examination** Temperature 104 F, pulse 126 per minute, respirations, 32 per minute. The sclerae were markedly icteric. There was no adenopathy. The liver descended one finger's breadth and the spleen two fingers' breadth below the right and left costal margins respectively.

**Laboratory Data** Blood counts: leukocytes 6100, erythrocytes 3,010,000, hemoglobin 9.3 Gm. differential polymorphonuclear neutrophils 45 per cent, band forms 21 per cent, monocytes 16 per cent, lymphocytes 18 per cent. Bone marrow: hyperplastic differential polymorphonuclear leukocytes 27.5 per cent, band forms 30.5 per cent, metamyelocytes 24.5 per cent, myelocytes 12.5 per cent, promyelocytes 1.5 per cent, plasma cells 2.5 per cent, reticulum cells 1.6 per cent, erythrocyte granulocyte ratio 1:2.5. The urine showed four plus albumin and four plus urobilinogen. The blood sedimentation rate was 125 mm. per hour. The total serum bilirubin was 2.5 mg. per cent. A roentgenogram of the chest revealed hilar adenopathy and increased markings extending down to the right lower lobe.

**Course** On April 17, 1947, the patient was started on a course of  $\text{HN}_2$  consisting of 6, 7, 8 and 6 mg administered on successive days. The first dose was followed within one half hour by a chill and within two hours by moderate nausea and vomiting. There were no reactions following the last three doses. Icterus was more intense on the third day of therapy. The patient became increasingly stuporous, lapsed into coma and died nine days after the institution of therapy.

**Postmortem Examination** There was a well-defined icterus of the skin and sclerae. The spleen and liver weighed 475 and 2240 grams respectively. There was extensive granulomatous infiltration within these organs as well as the tracheo-bronchial, paraortic and retroperitoneal nodes. Partial compression of the common bile duct resulted from an enlarged node at the head of the pancreas. Microscopic examination revealed numerous foci of necrosis in the liver with swelling and vacuolization of the reticulo-endothelial cells and marked hypoplasia of the bone marrow.

The progressively downhill course of this patient was probably accelerated by the administration of  $\text{HN}_2$  in the face of definite icterus. It is probable that the miliary necroses of the liver and hypoplasia of the bone marrow could be directly attributed to  $\text{HN}_2$  therapy.

**Ascites** Ascites was present in three cases. Partial relief was effected in two cases. Case 5, radioresistant, had marked ascites, pleural effusion, dyspnea, fever, anorexia and malaise.  $\text{HN}_2$  therapy and other supportive measures brought about a

satisfactory partial remission. Case 23 is illustrative of a partial response to vigorous therapeutic measures including HN<sub>2</sub>.

#### CASE 23

*V C* a 24 year old white female was first seen on January 7 1947. A diagnosis of Hodgkin's disease had been made two and one-half years prior to admission after a six month period of fever, sweats, hoarseness, adenopathy and splenomegaly. Roentgen therapy was only partially effective in reducing glandular enlargement. She had herpes zoster one year prior to admission. During the past three months she had developed progressive fatigue, anorexia, dyspnea, ascites and edema of the lower extremities.

The patient had a marked pancytopenia and hypoproteinemia and required frequent transfusions.

*Physical Examination* The patient was markedly emaciated. She had a right Horner's syndrome. There was generalized shorty adenopathy, marked ascites, hepatosplenomegaly and pitting edema of the lower extremities.

*Laboratory Data* Blood counts: leukocytes 2000, erythrocytes, 3,380,000, hemoglobin 56 per cent, platelets 267,020, reticulocytes 1.6 per cent, differential: polymorphonuclear neutrophils 32 per cent, band form, 26 per cent, metamyelocytes 3 per cent, monocytes 31 per cent, lymphocytes 7 per cent. Bone marrow hypercellular, differential: polymorphonuclear neutrophils 6.8 per cent, band forms 16.4 per cent, metamyelocytes 23.8 per cent, myelocytes 30.0 per cent, promyelocytes 1.4 per cent, myeloblasts 4.0 per cent, eosinophils 3.6 per cent, lymphocytes 0.4 per cent, plasma cells 0.2 per cent, reticulum cells 5.2 per cent, megakaryocytes plentiful, erythrocyte: granulocyte ratio 1:1. The urine showed two plus albumin. The Hinton test was negative. The total proteins were 4.4 gms. per cent, albumin 3.0 Gm., globulin 1.4 Gm.

*Course* The patient received five doses of HN<sub>2</sub> consisting of 3, 4, 5, 6 and 5 mg. administered on alternate days beginning January 11, 1947. She had moderate nausea and vomiting starting two hours after each injection and lasting 3 to 4 hours. The Horner's syndrome disappeared completely. Adenopathy and hepatosplenomegaly regressed partially. The patient received numerous supportive measures including intravenous blood, plasma, albumin, vitamins and paracentesis. The serum protein rose to 5.1 Gm. per cent. Leukocytes fell to 900 and penicillin was administered. The platelets rose to 410,000. Thirteen days after the initiation of therapy the bone marrow showed a marked decrease in cellularity and a shift of granulocytic elements to more mature forms. Improved appetite and general well being continued for about four months. In May 1947 she developed jaundice and severe epistaxis. A second course of HN<sub>2</sub> was instituted but ascites recurred and the patient went progressively downhill and died. Postmortem examination was not obtained.

This patient appeared to be in a terminal state upon admission and HN<sub>2</sub> was administered only after considerable hesitation especially since marked leukopenia was also present. However, following therapy the patient had a four month remission and in fact showed partial improvement of her pancytopenia. Rosenthal<sup>38</sup> has described the use of nitrogen mustard therapy with splenectomy in those cases having severe leukopenia. Splenectomy was found to be effective in raising the leukocyte level. Remissions tended to be of longer duration with this drastic procedure and the leukocyte count was not lowered. Experience with this form of combined therapy is as yet too limited to permit evaluation.

Case 22 showed ascites, hepatosplenomegaly, hydrothorax and fever.

Patients displaying ascites and extensive hepatic involvement have, on the whole, responded poorly to HN<sub>2</sub>. Boursnell, et al.<sup>39</sup> demonstrated the excretion of as much as 50 per cent of intravenously injected sulfur mustard into the bile of rabbits within one hour. With diffuse granulomatous infiltration biliary excretion is undoubtedly impaired, and the avenue of approach to involved areas obstructed. Roentgen therapy may be of some value in such cases.

## *Neurologic Involvement*

### *Intraspinal*

Intraspinal involvement has been attributed to the following pathogenetic mechanisms (1) extension from retroperitoneal and posterior mediastinal granulomatous tissue via the intervertebral foramina into the epidural space, (2) extension from an involved vertebra, or compression from collapsed vertebral bodies, (3) mechanical obstruction of blood vessels within the intervertebral foramina or just outside the cord, causing diffuse myelomalacia, and (4) toxic myelitis.<sup>34-36</sup> Pressure from lesions extending from involved vertebrae was present in one treated case (case 20) and in one untreated case (case 6). In the other cases, there was probable extension via the intervertebral foramina. Thromboses of blood vessels may well have been a contributing factor in some cases.

**Spastic Paraplegia** During the course of our observations, 5 patients developed spastic paraplegias. In 3 cases this developed terminally and we did not have the opportunity to treat them with nitrogen mustard. The other 2 cases are described in detail. The results of treatment with HN<sub>2</sub> were of only partial and temporary value.

### CASE 8

**G. S.** a 31 year old housewife was first seen in July 1946. She had developed cough, pruritus, cervical and axillary adenopathy and splenomegaly in 1944. Roentgen therapy induced a six year remission. In January 1946 she noted the onset of painful swelling of the left arm and breast. Cough, dyspnea and fatigue were present. In March 1946 Roentgenograms of the chest showed a massive hydrothorax and bilateral hilar adenopathy. Two thoracotomies brought about considerable relief of dyspnea. This was followed by roentgen therapy to the mediastinum with complete resorption of the left thoracic fluid and regression of hilar adenopathy. The edema of the left breast and arm persisted. Three weeks later, however, a recurrence of the pleural fluid and mediastinal adenopathy was noted and the patient was referred for nitrogen mustard therapy.

**Physical Examination.** The patient had a marked radiodermatitis of the left supraclavicular area. There were induration and edema of the left breast and upper extremity, a left Horner's syndrome as well as signs of pleural thickening over the left upper chest. There were no palpable glands, liver and spleen were not enlarged. Neurologic examination was negative.

**Laboratory Data.** Blood counts: leukocytes 7600, erythrocytes 3,540,000, hemoglobin 11.3 Gm, platelets 1,176,630, reticulocytes 1.3 per cent, differential: polymorphonuclear neutrophils 81 per cent, monocytes 7 per cent, lymphocytes 12 per cent. The urine was negative. The Hinton test was negative. Roentgenogram of chest showed enlarged hilar masses.

**Course.** On July 5, 1946 the patient was started on a course of 4 doses of HN<sub>2</sub> consisting of 4, 5, 6 and 7 mg. Chills and severe nausea followed each dose.

There followed a moderate regression of the edema of the left arm and breast and complete resolution of both hilar masses. In October 1946 the patient developed a spastic paraplegia and fecal and urinary incontinence. Combined roentgen therapy (1025 r to the lower cervical and upper thoracic spine) and HN<sub>2</sub> (24 mg) were administered. Severe nausea and vomiting followed each injection of the latter. During the course of the next three months incontinence completely disappeared and the patient could walk with assistance. Horner's syndrome persisted.

In February 1947, the patient again developed a spastic paraplegia. Lumbar puncture demonstrated the presence of a partial dynamic block and a spinal fluid protein of 120 mgs per cent. A third course of HN<sub>2</sub> was administered on four successive days (5, 6, 7 and 8 mg). Spinal fluid dynamics returned to normal and protein level fell to 60 mgs per cent. Roentgen therapy to the lower cervical and upper thoracic spine (725 r) produced no further effect upon the spinal fluid. There followed a gradual improvement in the use of both lower extremities.

In May 1947 the patient developed subcutaneous nodules over the left upper chest. These soon ulcerated and became secondarily infected. Progressive paralysis of the lower extremities resulted in a complete spastic paraplegia and urinary incontinence. The edema of the left arm became especially painful. All forms of therapy were refused. In September 1947 a large sacral ulcer developed and became secondarily infected. The patient ran a fever which did not respond to penicillin therapy. She was continuously sedated with large doses of morphine and pantopon. Death occurred on September 27, 1947. Postmortem examination was not obtained.

#### CASE 24

J. F. K., a 27 year old white male first noted the presence of left cervical adenopathy in December 1943. A biopsy revealed the presence of Hodgkin's granuloma. In November 1945 mediastinal involvement was noted. In March 1946 left inguinal glands appeared. Cervical glands recurred in July 1946. Roentgen therapy induced complete regression of enlarged glands. Complaints of anorexia, weakness, nausea, vomiting, epigastric and flank pain were relieved by roentgen therapy to the abdomen and back. In October 1946 the patient complained of left upper quadrant pain and paresthesias of the lower extremities. The upper abdominal pain subsided with x-ray therapy. Complete paraplegia and urinary incontinence developed in January 1947.

*Physical Examination.* The patient appeared chronically ill. He had enlarged cervical, left axillary, inguinal and femoral nodes. A lime-sized mass was palpable in the lower abdomen. The spleen was two fingers breadth below the left costal margin. There was a large sacral ulcer. Neurologic examination revealed a spastic paraplegia and hypesthesia from the level of D12. The patient had both urinary and fecal incontinence.

*Laboratory Data.* Leukocytes 18,900; erythrocytes 3,280,000; hemoglobin 72 per cent; differential polymorphonuclear neutrophils 89 per cent; monocytes 1 per cent; lymphocytes 10 per cent. The urine showed a trace of albumin and numerous white cells. The Mazzini test was negative.

*Course.* Two courses of HN<sub>2</sub> were administered, one beginning January 2, 1947 (22 mg.) and the other February 17, 1947 (22 mg.). This was followed by roentgen therapy (3200 r) over the lower dorsal and upper lumbar spine. The neurologic status however remained unchanged. Cervical and inguinal glands appeared about one month later. In June 1947 the patient developed edema of the left leg and scrotum which was unrelieved by mercurhydrin. Bladder incontinence required constant tidal drainage. In August 1947 enlarged cervical glands appeared and the patient had considerable dysphagia. He received 4 mg. HN<sub>2</sub> on August 18. The following day at the start of the saline infusion for the administration of nitrogen mustard he became dyspneic and cyanotic and complained of sudden blindness. His face became puffy and neck veins distended. Oxygen and morphine were administered with gradual improvement. A friction rub was heard at the left base twenty-four hours later. One month later a cutaneous ulcer developed at the base of the penis due to pressure from the paraplegic position. The patient died on October 17, 1947.

At autopsy there was granulomatous infiltration of cervical, axillary, inguinal, retroperitoneal, celiac, pancreatic and mesenteric lymph nodes, the spleen and liver as well as infiltration into the psoas muscle, kidneys, adrenals, bladder, pancreas and left lung. There were bilateral pyonephroses and pyoureteritis. A purulent cystitis was present. The lower thoracic portion of the spinal cord was surrounded by an epidural cuff of firm gray tumor 3 cm. in thickness. The left half of the cord was greatly compressed. The tumor extended through the dura and pia arachnoid directly into the substance of the cord. There was degeneration of the posterior and lateral tracts of the spinal cord. The vertebral marrow was entirely replaced by necrotic tissue. There was active hematopoiesis in the costal, sternal and calvarial marrow.

Therapy was instituted three weeks and three and one-half months after the initial symptomatology in cases 8 and 24 respectively. In the latter case, irreversible cord changes were undoubtedly present at the time of treatment. The former had a partial remission following combined therapy. The shorter interval between onset of symptoms and therapy is probably responsible for the difference in the results.

obtained. Secondary myelomalacia of the cord due to pressure and thrombosis of vessels is an irreversible process.

**Pain.** Pain was a prominent presenting symptom in three patients who showed evidence of intraspinal involvement of Hodgkin's disease.

*In case 25* the initial manifestation of the disease was in the form of agonizing low back pain radiating down the right leg. Roentgenogram of the spine revealed the presence of a destructive lesion in the twelfth dorsal vertebrae. A laminectomy performed one year later revealed an infiltrative mass involving the seventh, eighth, ninth and tenth dorsal spinous processes, laminae and pedicles as well as an extradural mass. Roentgen therapy did not relieve the pain. Because of the excruciating character of the pain the patient required large doses of morphine and demerol to which he became addicted. The patient subsequently had two convulsive seizures with shooting pains down both arms. Four courses of HN<sub>2</sub> were administered following which he developed complete remissions from the agonizing pain for the periods of 31, 43, 28 and 21 days respectively. The second course was combined with roentgen therapy. Further HN<sub>2</sub> had to be discontinued because of hematemesis. The patient died suffering extreme back pain radiating down both legs. At autopsy the extradural space from the lumbar to the upper cervical area was filled with tumor.

*Case 38* was completely relieved of pain following the first and partially relieved following the second course of nitrogen mustard.

The following case, showing remarkable pain relief following HN<sub>2</sub> therapy, is described in detail.

#### CASE 10 (FIGURE 10)

*B. D.* a 38 year old white male was first seen in October 1946. He had discovered a mass in the left axilla two and one half years previously and the diagnosis of Hodgkin's disease had been made following biopsy. Roentgen therapy was then administered to the left axilla, left supraclavicular region and mediastinum. In September 1944 the patient developed fever, night sweats and right axillary adenopathy. A submental gland appeared in December 1944.

Roentgen therapy was administered on these and subsequent occasions with progressively increasing radioresistance. The patient developed marked fatigue, lassitude, night sweats and anorexia.

**Physical Examination** (October 22, 1946) The patient was moderately pale. A large mass was present in the eleventh left intercostal space. There was no cervical axillary, or inguinal adenopathy. The liver was felt three fingers breadth below the right and the spleen five fingers breadth below the left costal margin.

**Laboratory Data.** Blood counts: leukocytes 15,650; erythrocytes 3,390,000; differential polymorphonuclear neutrophils, 44 per cent, band forms 10 per cent, lymphocytes 34 per cent, monocytes 10 per cent.

**Course.** The patient received four doses of HN<sub>2</sub> consisting of 4, 4, 5 and 6 mg. administered on alternate days. Moderate nausea and vomiting followed each injection and lasted for three to five hours. Within a few days the patient had a marked increase in vitality, an increased appetite and began to gain weight. The mass in the eleventh intercostal space and the hepatosplenomegaly regressed completely. The leukocyte level dropped to 3,950. This remission lasted for two and one half months. At that time the patient noted the presence of enlarged preauricular glands. On examination he was found to have generalized adenopathy, recurrent eleventh left intercostal mass and hepatosplenomegaly. A second course of HN<sub>2</sub> was instituted on December 26, 1946 in the form of weekly and biweekly injections. The nausea and vomiting were of such severity that further attempts at prophylactic therapy had to be discontinued. Adenopathy and hepatosplenomegaly regressed completely.

In February 1947 the patient noted the onset of headache and irritability. This was soon followed by intermittent pain in the right quadriceps muscle, severe sweats and anorexia. About one month later the patient complained of low back pain radiating down the right extremity, aggravated by coughing, sneezing and straining at stool. Neurologic examination was essentially negative. The pain shifted to the left lumbar area and radiated to the left hip and left thigh anteriorly. Roentgen therapy (300 r) to



the lumbar spine had no effect. The pain localized at L<sub>3</sub> and became progressively more intense. A lumbar puncture revealed a complete dynamic block, xanthochromic fluid and 872 mg per cent spinal fluid protein. Other physical findings and hematologic data are depicted in figure 10.

Beginning April 25, 1947 the patient received daily injections of 4, 5, 6 and 7 mg HN<sub>2</sub>. Moderate nausea and vomiting followed each dose. Within twelve hours after the first dose 90 per cent of the pain had subsided and moderate reduction in preauricular adenopathy was noted. The pain was almost completely relieved at the conclusion of therapy. A repeat lumbar puncture was performed on the following day and revealed normal dynamics, clear fluids and 39 mg per cent spinal fluid protein. Sweats and hepatosplenomegaly subsided completely. The usual fall in the leukocyte level occurred.

In July 1947 the patient noted a recurrence of weakness, anorexia and dizziness. On examination he was found to have marked pallor and hepatosplenomegaly. Blood counts were as follows: leukocytes

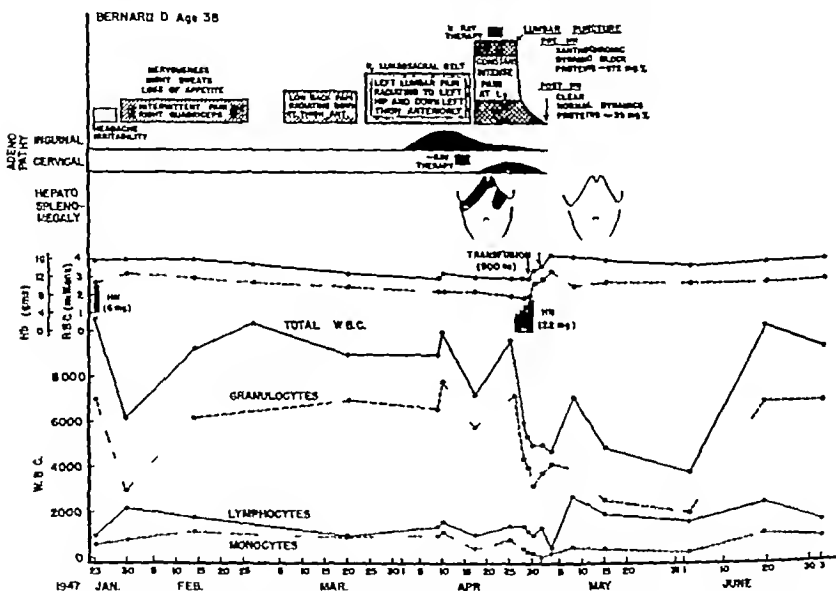


FIG. 10—EFFECTS OF HN<sub>2</sub> IN PATIENT WHO DEVELOPED AGONIZING LOW BACK PAIN AND EXTRADURAL INVOLVEMENT WITH HODOKIN'S DISEASE (CASE 10)

6000, erythrocytes 2,010,000, hemoglobin 6.9 Gm, reticulocytes 6.6 per cent, platelets 303,000 differential, normal. The blood sedimentation rate was 68 mm per hour. The urine urobilinogen was positive 10 x 320 dilution. The fourth course of HN<sub>2</sub> was started on August 9, 1947 and consisted of daily doses of 5, 6, 7 and 8 mg. The usual reactions of nausea and vomiting followed each dose. 1000 cc of whole blood were given to correct the anemia. There followed a marked improvement in anorexia and sweats. The hepatosplenomegaly subsided completely. The leukocyte level fell to 2900.

The remission lasted until October 20, 1947 when the patient again noted the onset of fatigability and anorexia. On examination he was found to have moderate pallor, preauricular and submental adenopathy and hepatosplenomegaly. The erythrocyte count had fallen from 4,040,000 to 3,300,000 with corresponding hemoglobin levels of 11.3 and 9.3 Gm, respectively. A fifth course of HN<sub>2</sub> was started on October 23, 1947 consisting of 5, 6, 7 and 8 mg administered on alternate days. The patient had a prompt improvement in general well being as well as complete regression of adenopathy and hepatosplenomegaly. The leukocyte level dropped to 3600. The platelet count rose to 404,500.

Following each of the five successive courses of HN<sub>2</sub> this patient demonstrated an unusual sensitivity to nitrogen mustard therapy with objective signs of improvement occurring as early as 12 hours after the initial dose. The response of pain and the regression of the intraspinal tumor were indeed remarkable. The consistent fall in erythrocyte and hemoglobin levels and reticulocytosis were quickly corrected with HN<sub>2</sub> therapy.

In this group presenting pain as the predominant symptom the results were far more striking than in those cases showing paraplegia, probably because pain is an early sign of intraspinal involvement and may therefore cause the patient to seek help before irreparable spinal cord damage has taken place. Thus pain was completely relieved in 71.4 per cent and partially relieved in 28.6 per cent of the cases.

### *Peripheral*

*Paralysis of the Upper Extremity* In 2 cases, paralysis of the upper extremity, secondary to pressure upon the brachial plexus was present. In neither case was nitrogen mustard effective in relieving the paralysis. Case 1, had a large mass filling the entire left side of the neck. Case 8 had extensive scar tissue in the supraclavicular region which resulted from previous intensive roentgen therapy. The cervical mass in former case showed partial regression but sudden death occurred before any improvement in the paralysis could be noted.

*Pain* Back pain in the absence of specific intraspinal disease was present in 7 patients. Complete subsidence of pain in all cases followed nitrogen mustard therapy. It is probable that dorsal root compression by granulomatous tissue was quickly relieved before irreversible changes had occurred.

*Horner's Syndrome* Seven patients having eleven administrations of HN<sub>2</sub> had Horner's syndrome. No change followed therapy in nine of eleven administrations.

### *Osseous Involvement*

Roentgenograms of the skeletal system revealed lesions in 6 patients. Vertebral lesions were present in 4 cases, pelvic and vertebral lesions in 1 case, and pelvic lesions alone in 1 case. A destructive lesion of the sternum was present in 1 case. Despite successful clinical remissions following nitrogen mustard therapy the destructive lesions as visualized roentgenologically showed no improvement. This lack of response may be due to the inhibitory effect of the nitrogen mustards upon osteoblastic and other enzymatic activities necessary for osseous regenerations.

## EFFECTS ON HEMATOLOGIC CONSTITUENTS

### *Peripheral Blood*

*Erythrocytes* Figure 11 illustrates the hematologic changes which followed HN<sub>2</sub> therapy. Fifty-eight and five-tenths per cent of cases showed a well defined decrease in the erythrocyte level. This was manifest within five to six days after the initiation of treatment, and persisted until the twenty-first to twenty-fifth day, after which a gradual increase to normal levels occurred. The maximum reduction in the erythrocyte count was 16.2 per cent. In 18.8 per cent of cases, erythrocytes rose

following therapy. The average rise was 12.2 per cent on the ninth to tenth day and 23.3 per cent on the twenty-sixth to thirtieth day. Twenty-two and seven-tenths per cent of cases showed slight if any change in red cell count. The routine examination of all peripheral blood films failed to reveal any striking morphologic changes in the red blood cells.

It is possible that the effect on red cell count may be due to a direct action of the chemical upon the circulating red cell. Boursnell, et al.<sup>30</sup> have demonstrated

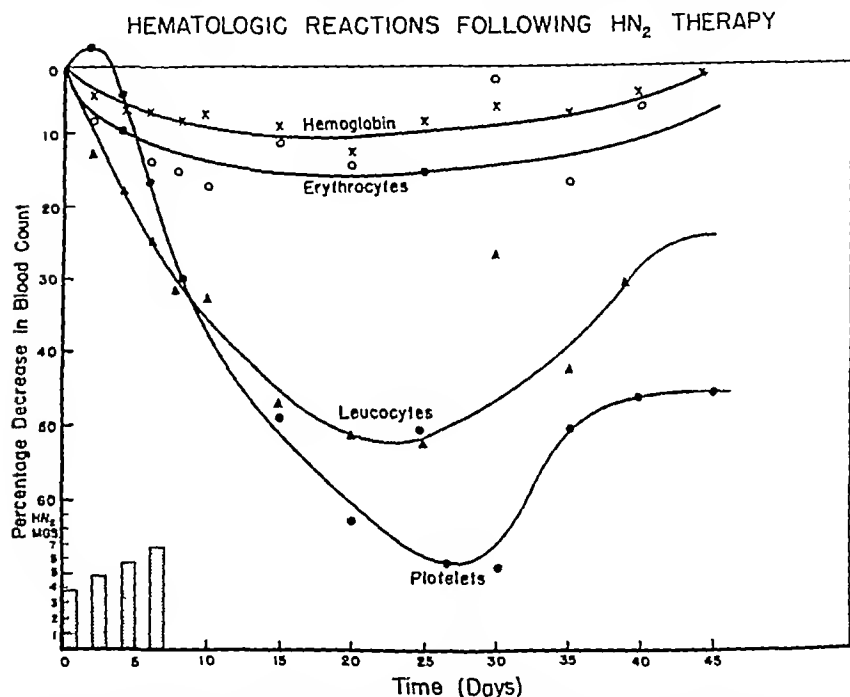


FIG. 11.—REDUCTION IN THE VARIOUS HEMATOLOGIC CONSTITUENTS FOLLOWING A COURSE OF  $\text{HN}_2$  THERAPY. The platelet reduction as pictured above occurred in only 20 per cent of the cases. The most constant effect was on the leucocytes, more particularly on the granulocytic elements.

in rabbits, that one-third of the injected radioactive sulfur mustard remains affixed to the red cells. Increased urobilinogen excretion into the feces has been reported by Jacobson<sup>8</sup> and Urteaga<sup>30</sup> following the administration of nitrogen mustard. Serial serum bilirubin studies, performed in many of our cases, revealed no change. No spherocytosis or altered osmotic fragility of the red cells could be demonstrated.

Case 10, with each relapse, showed a marked fall in erythrocyte and hemoglobin levels and developed a spherocytosis and reticulocytosis. Following each course of nitrogen mustard therapy the erythrocyte and hemoglobin levels rose and spherocytes and reticulocytes diminished.

**Hemoglobin** A parallel fall in the hemoglobin level to that noted above occurred in 59.0 per cent of cases. A 7.1 per cent reduction was present on the fifth day, and a 12.1 per cent reduction on the fifteenth to twentieth day. Gradual improvement followed. In 41.0 per cent of cases, a rise in the hemoglobin levels to a maximum of 19.3 per cent on the twenty-first to twenty-fifth days was noted.

**Reticulocytes** Eighty-five and one-tenth per cent of cases showed a depression in the reticulocyte level following nitrogen mustard therapy. This was maximal on the sixth to tenth day (0.0 to 0.2 per cent).

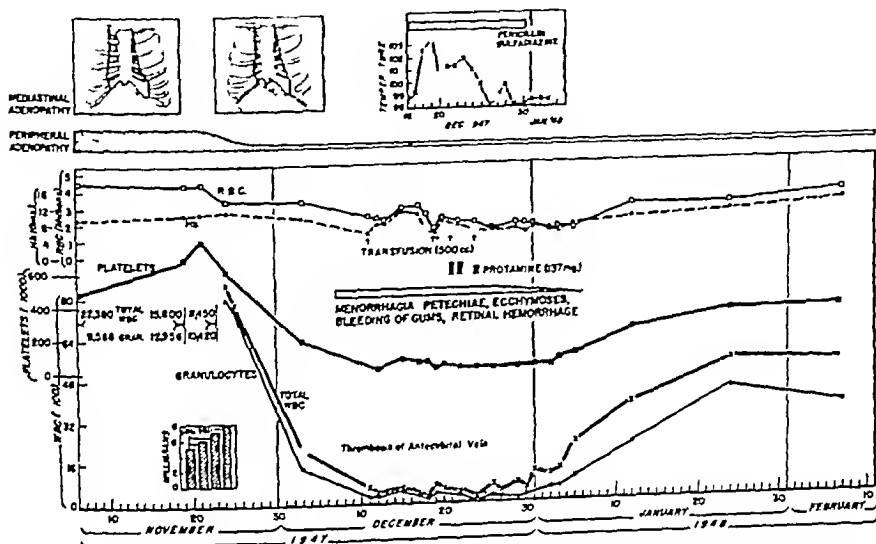


FIG. 12.—SEVERE HEMATOLOGIC COMPLICATIONS FOLLOWING ADMINISTRATION OF 18 MG  $\text{HN}_2$  AND 8 MG  $\text{HN}_2$  (CASE 49). The various features of aplasia of the marrow developed with severe anemia, granulocytopenia and thrombocytopenia. Hemorrhagic manifestations were severe. Patient recovered after a very stormy course and had an excellent remission.

**Leukocytes** A fall in the leukocyte level occurred in 87.7 per cent of cases. Those patients with initial leukocyte counts ranging from 4000 to 15,000 tended to develop leukopenic levels, while those ranging between 15,000 and 27,250 tended to fall to normal levels. The maximal fall in the leukocyte count occurred on the twenty-first to the twenty-fifth day after the initiation of treatment and was followed by a gradual return to normal levels on the thirty-sixth to fortieth day.

Five cases (6.9 per cent) with initial leukopenias after a slight decrease in the leukocyte count showed a progressive increase beginning on the sixteenth to twentieth day. Case 23 had an initial leukocyte count of 2600 which fell to 900 on the eleventh day and subsequently rose to 6920 on the thirty-third post-therapy day.

Case 49 showed the most marked leukocyte depression falling from 22,350 to 600 and lasting for forty days (fig. 12). This was associated with a corresponding reduction in the erythrocytes and platelets and progressive bone marrow hypo-

plasia. The etiologic role of tris-mustard in the production of this reaction is discussed below.

The decrease in the leukocyte count was predominately a reflection of the simultaneous decrease in granulocytes (figs 5, 9, 10, 12). Lymphocytes and monocytes showed moderate reductions only when their initial levels were high. Repeated examinations of the blood films revealed no qualitative changes in any of the white cell elements.

*Platelets* The platelet level was affected in only 20.5 per cent of the cases. In these cases, an average reduction of 69.4 per cent was present on the twentieth to thirtieth day following which a gradual increase occurred. Two patients with initially low platelet counts after a slight depression showed increases of 43.8 and 54.4 per cent.

### *Hemorrhagic Manifestations*

Upon the usual therapeutic schedule, 3 patients developed hemorrhagic manifestations following one or more courses of  $\text{HN}_2$ . Case 20, who had received a total of eight courses, developed moderate bleeding of the gums following her last course. Severe hematemesis followed the third and fourth doses of the fourth course in Case 28 and well as the fifth dose of the fifth course. Hematemesis occurred terminally eleven days after the first course in Case 22. The most severe hemorrhagic complications due to marked thrombocytopenia, occurred in Case 49 who received 18 mg of the tris compound ( $\text{HN}_3$ ) and 8 mg of  $\text{HN}$ . Her case is described in detail.

### CASE 49 (FIGURE 12)

*M. S.* a 19 year old white female first noted the presence of a right supraclavicular mass in October 1947. Within two weeks she began having severe night sweats and fever. Other glands appeared in the left cervical and axillary regions. The biopsy showed features of both Hodgkin's granuloma and sarcoma. The patient was first seen about one month after onset at which time she complained of cough.

*Physical Examination* The patient was moderately pale. There was a large left axillary mass 6 cm in diameter. There were numerous bean-sized axillary and cervical glands. Supracardiac dullness was increased. The liver and spleen were not palpable.

*Laboratory Data* Blood counts: leukocytes 15,180 erythrocytes 4,310,000 hemoglobin 10.4 Gm reticulocytes 0.5 per cent platelets 689,600 differential polymorphonuclear neutrophils, 82 per cent eosinophiles 3 per cent monocytes 4 per cent lymphocytes 11 per cent. Bone Marrow hyperplastic differential band forms 36 per cent metamyelocytes 22.5 per cent, myelocytes 11.6 per cent plasma cells, 1.2 per cent megakaryocytes markedly increased normoblasts A 0.8 per cent B 2.8 per cent, C, 4.0 per cent. The urine was negative. The Hinton test was negative. The blood sedimentation rate was 95 mm per hour. The total blood proteins were 7.1 Gm per cent albumin 4.4 Gm per cent, globulin 2.7 per cent. A roentgenogram of the chest showed large mediastinal and hilar masses.

*Course* The patient received three doses of  $\text{HN}_2$  (5, 6 and 7 mg respectively) and one dose of  $\text{HN}$  (8 mg) on alternate days beginning November 18, 1947. There was a strikingly rapid regression of cervical and axillary glands. On the fifth day following the initiation of therapy the mediastinal masses regressed 60 to 70 per cent. The patient's hematologic course is shown in figure 12. A marked pancytopenia with extreme thrombocytopenia developed. Severe menorrhagia, petechiae, ecchymoses, bleeding of gums and a retinal hemorrhage occurred eighteen days after the initiation of therapy. Thromboses of the right and left antecubital veins were present. Serial bone marrow aspirations revealed progressive hypoplasia (fig 13). The patient ran a febrile course for ten days. During this time she received penicillin and sulfadiazine. A total of 3500 cc of fresh whole blood was administered. Protamine 137 mgs was

administered intravenously but without apparent effect upon the hemorrhagic manifestations. These subsided spontaneously with an improvement in the platelet count. Definite evidence of bone marrow regeneration was noted on January 5, 1948.

The  $\text{HN}_2$  administered to this patient was undoubtedly largely responsible for the severity of the hemorrhagic complications. This form of nitrogen mustard was found to produce unusually severe depressions of leukocyte, erythrocyte and platelet levels. Thromboses of injected veins were likewise more common. Further use of tris-mustard appears to be unwarranted.

### *Bone Marrow*

Serial bone marrow studies were performed in 11 cases of Hodgkin's disease treated with nitrogen mustard. Within twenty-four hours after the initiation of nitrogen mustard therapy the clumps of marrow began to show a decrease in size and cellularity. Fat-spaces were increased.

Polymorphonuclear neutrophils showed hypersegmentation. Erythropoiesis was suppressed. Within two to four days there was a reduction in the number of myelocytic cells and a relative increase in the number of more mature forms. Bizarre, distorted myelocytes, metamyelocytes, polymorphonuclear neutrophils and megakaryocytes were noted with moderate frequency. Marked hypoplasia of the bone marrow followed nitrogen mustard therapy in 7 cases. The serial bone-marrow changes obtained in 1 case are shown in fig. 13. Case 46 showed a marked decrease in cellularity within 24 hours after the initiation of therapy. Increasing hypoplasia was found two days later. Bone marrow regeneration was noted six days after the cessation of therapy. The pretherapy bone-marrow of case 35 was markedly hyperplastic. Severe hypoplasia was present nine days after the initiation of treatment.

A moderately active bone-marrow was present in Case 16 who died fifteen days after the completion of the last course of nitrogen mustard. Hyperactive marrows were found at autopsy in 2 cases (Cases 15 and 24) who died five and ten months, respectively, after their last course of therapy.

Suppression of erythroid activity was noted within twenty-four hours after the initiation of  $\text{HN}_2$  therapy. No immediate reflection of this depression was noted in the peripheral erythrocyte and hemoglobin levels, in all probability because of the normal red cell survival time of one hundred and twenty days.

Bloom and Bloom<sup>40</sup> showed that the chick erythroblast was the most sensitive cell in the marrow following the administration of x-ray therapy.

Suppression of granulopoiesis was noted within two to four days. The fall in the peripheral leukocyte level occurred shortly thereafter reaching a maximal leukopenia on the twenty-fifth day. This prompt reflection of an effect on the marrow is undoubtedly due to the short survival time of the leukocyte in the peripheral blood.

Megakaryocytes proved to be the most resistant of all marrow elements and platelet reduction occurred in only 20.2 per cent of cases.

Except for terminal cases dying shortly after their course of nitrogen mustard therapy no cases of irreversible aplasia of the marrow were encountered in this

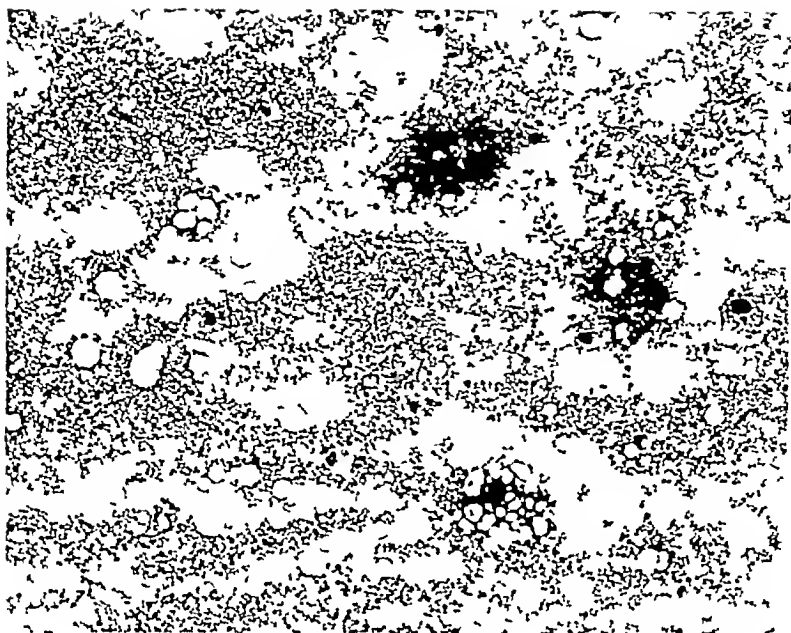


FIG 13 —HYPOPLASTIC RESPONSE OF BONE MARROW FOLLOWING INJECTION OF 18 MG TRIS (B CHLOROETHYL) AMINE AND 8 MG METHYL BIS (B CHLOROETHYL) AMINE (CASE 49)  
(a) PRIOR TO INITIATION OF THERAPY

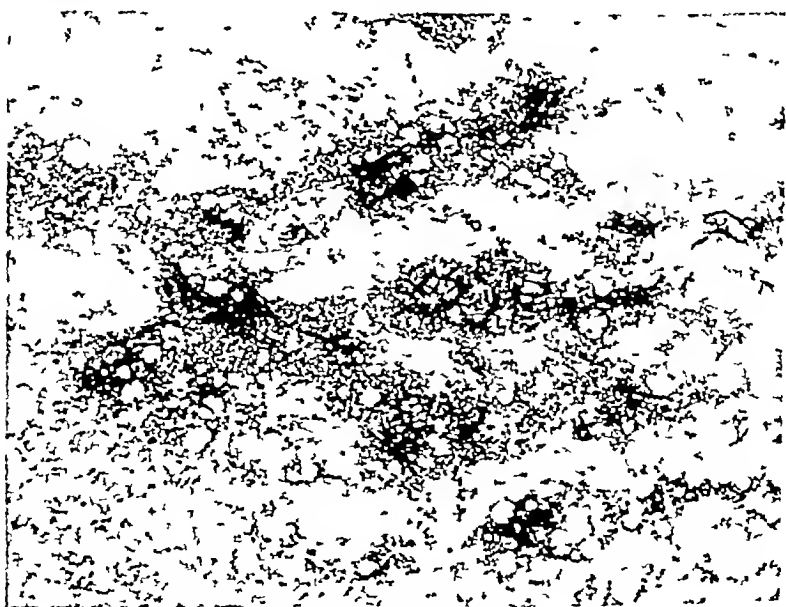


FIG 13 —(b) EIGHT DAYS AFTER INITIATION OF THERAPY

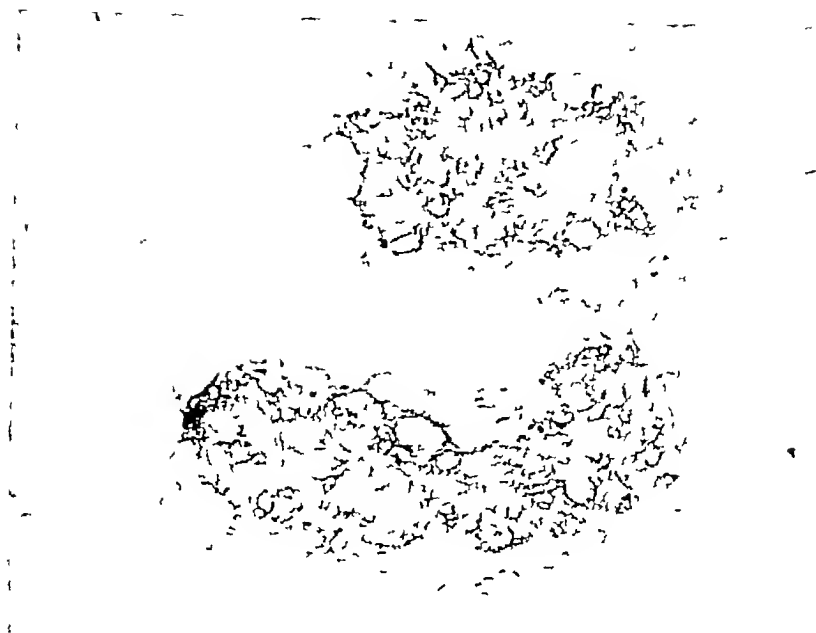


FIG 13 —(c) TWENTY DAYS AFTER INITIATION OF THERAPY



FIG 13 —(d) THIRTY EIGHT DAYS AFTER INITIATION OF THERAPY



series. However, we have been informed of such instances from other clinics where a higher dosage schedule of  $\text{HN}_2$  and more frequent institution of therapy have been in vogue. Our experience indicates that the tris compound has a much greater cytotoxic effect upon the bone marrow than does the methyl Bis (B chloroethyl) amine.

In experimental animals, the rapidity of the cytotoxic action of the nitrogen mustard has been demonstrated by Karnofsky et al.<sup>29</sup> This occurs within a period of five minutes after injection. The fixation of radioactive sulfur mustard to the bone marrow was shown by Boursnell et al.<sup>30</sup> Kindred<sup>41</sup> studied the reaction of the femoral bone marrow of the albino rat to sulfur and three nitrogen mustard preparations. A marked suppression of erythroid and granulocytic elements was noted two days after injection. Mitotic activity was diminished. Megakaryocytes showed some signs of injury but no reduction in number. Reticulum cells and plasma cells were unaffected. Similar results were obtained in dogs, rabbits,<sup>42</sup> and in mice.<sup>37</sup>

Severe aplasia of the bone marrow following mustard gas poisoning was reported in 6 fatal cases by Krumbhaar and Krumbhaar<sup>6</sup> in 1919. Spurr, et al. using the marrow aspiration technique<sup>32</sup> found a more prolonged depression of the marrow and a less rapid return to normal than noted in our cases. Block et al.<sup>43</sup> studied the serial marrow changes histopathologically. The atrophic stage was between eight and twenty days after initiation of  $\text{HN}_2$  therapy. In the post-mortem findings reported by Spitz,<sup>44</sup> severe marrow hypoplasia was noted following a cumulative dose of 0.5 to 0.6 mg./kilo of  $\text{HN}_2$  administered eight days prior to death.

Barron et al.<sup>45</sup> showed that the addition of choline, dimethyl amino ethanol and methionine to bone marrow in vitro protected it from the inhibition of respiration by the nitrogen mustards.

### *Lymph Nodes*

Serial lymph node aspiration were performed in six treated cases. The typical appearance of the lymph node aspiration in Hodgkin's disease is shown in fig. 14a. This is characterized by a pleomorphic cellular pattern consisting of lymphocytes, polymorphonuclear neutrophils, eosinophils, plasma cells, reticulum cells, and Dorothy Reed cells. Within a period of twenty-four hours after the initiation of therapy there was a decrease in cellularity and pyknosis and smudging of lymphocytic cells. Four days after the initiation of therapy, these findings were more marked (fig. 14b). Polymorphonuclear neutrophils showed vacuolation and hypersegmentation. Reticulum cells were bizarre and degenerate and showed frequent vacuolation.

No change was noted in the lymph node aspirations of a case of Hodgkin's sarcoma (Case 46) who was resistant to treatment. Case 49, having some features of both Hodgkin's granuloma and sarcoma showed a marked reduction in the pleomorphism present before therapy with large numbers of sarcoma cells still present after therapy. The lymph nodes of Case 30, who died from an exanguinating gastrointestinal hemorrhage twenty-four hours after receiving 4 mg. of  $\text{HN}_2$ , showed marked pyknosis and diminished mitotic activity. Figure 15 shows a multi-

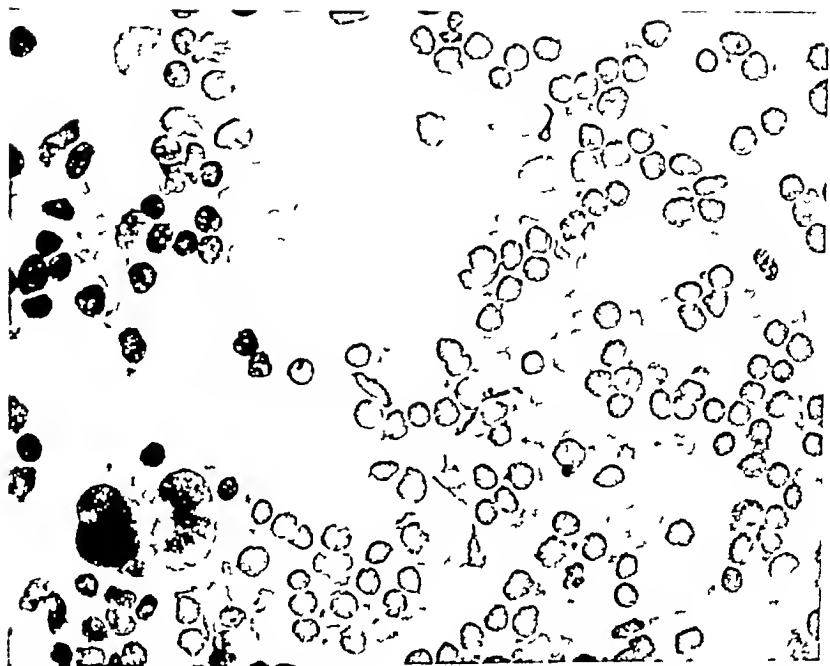


FIG. 14—EFFECTS OF NITROGEN MUSTARD ON LYMPH NODE OF CASE OF HODOKIN'S DISEASE (CASE 17)  
(a) PRIOR TO INITIATION OF THERAPY

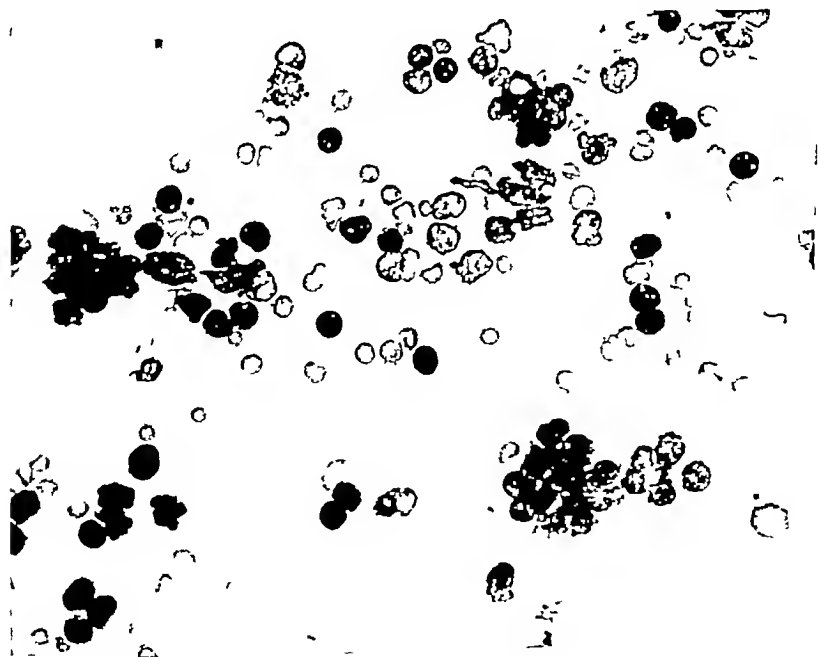


FIG. 14—(b) SAME PATIENT FOUR DAYS AFTER INITIATION OF THERAPY

ary focus of necrosis within a lymph node obtained at postmortem examination seven days after the institution of nitrogen mustard therapy (Case 28)

Similar results were noted by Block et al.<sup>43</sup> and Spitz.<sup>44</sup> Focal necrosis of the splenic pulp was reported by the latter following cumulative doses of 0.5 to 0.8 mg./kilo, administered seven to eight days before death. Kindred<sup>45</sup> showed marked lymphoid atrophy and lymphocytic degeneration in the albino rat on the second postinjection day. Similar changes were present in the thymus and the spleen. The peripheral lymphocytopenia coincided with decreased production within



FIG. 15.—LYMPH NODE OBTAINED AT POST MORTEM EXAMINATION SEVEN DAYS AFTER INITIATION OF NITROGEN MUSTARD THERAPY SHOWING A FOCUS OF MILIARY NECROSIS (CASE 41)

lymphoid organs rather than a direct effect upon the peripheral lymphocyte. Mice and rabbits showed essentially the same changes.<sup>27</sup>

#### *Liver*

Case 30 who died one day after a single injection of 4 mg. HN<sub>2</sub> showed an increase in the number of polymorphonuclear cells within the sinusoids. Milium foci of necrosis of the liver were noted at postmortem examination in 3 cases (Cases 16, 28 and 35), who died nine, ten and nineteen days, respectively, after the initiation of therapy. The liver cells showed extensive necrosis with very little leukocytic reaction (fig. 16). Four cases who died from fifty-four days to eight months after their last course of treatment showed no evidence of such milium foci of necrosis.

Nitrogen mustard appears to exert a karyolytic effect upon liver cells. Polymor-

phonuclear infiltration is present within twenty-four hours. Resolution of the necrotic foci probably takes place between nineteen and fifty-four days after the institution of therapy. Bournsnel, et al.<sup>20</sup> demonstrated the ability of the rabbit liver to concentrate as much as 50 per cent of the injected radioactive sulfur mustard within the bile within one hour. It is probable during this time that the hepatotoxic effect occurs. Zimmerman<sup>18</sup> reported focal necroses in the liver of cats after the oral administration of nitrogen mustard.



FIG. 16.—MILIARY FOCUS OF LIVER NECROSIS IN CASE OF HODGKIN'S DISEASE WHO DIED NINETEEN DAYS AFTER LAST COURSE OF NITROGEN MUSTARD THERAPY (CASE 16)

#### COMMENT

We prefer to classify Hodgkin's disease as a malignant proliferation of reticulum cells originating in lymphoid tissue, perhaps as a result of various stimuli including infections. The Sternberg-Reed giant cell, the type cell of this proliferative process, may represent a malignant type of reticulum cell. As with all neoplastic processes, Hodgkin's disease varies greatly in growth potentiality from case to case. In the most benign types giant cells are scarce and a tendency to fibrosis is marked. In the most highly malignant (Hodgkin's sarcoma), giant cells are

common and but little tendency to fibrosis is present. Dissemination of the disease ordinarily occurs by way of the lymphatic channels and by contiguity, and only rarely by way of the blood stream. Whatever the growth potentiality of the disease may be in a given case its course is relentlessly progressive. From peripheral lymph nodes, it extends to the mediastinum and the spleen. Thence, it spreads to visceral organs and constitutional symptoms of fever, night sweats, increasing weakness and itching appear. Terminally, it may block large lymphatic channels and cause huge tumor masses in various parts of the body.

The course of Hodgkin's disease may be terminated by complete extirpation of a single node or a small group of nodes if this is the only source of the disease. Such a successful end result is extraordinarily rare since once the diagnosis has been made, the disease has already spread. X-ray therapy has been used for many years to shrink the tumor masses and produce remissions. We have been impressed with the better results obtained in early cases by *drastic* x-ray therapy as opposed to the use of just enough x-ray to induce a reduction in lymph node size to normal. Sooner or later, despite x-ray therapy, new lymph node masses develop and constitutional symptoms become marked. At this point, x-ray therapy often has but little effect. The use of  $\text{HN}_2$  has to our mind revolutionized at least this phase in the treatment of the disease.

In the course of our studies it became apparent that  $\text{HN}_2$  is a valuable therapeutic tool in the treatment of Hodgkin's disease, in many cases, indeed, it presents distinct advantages over the more standard form of therapy by x-ray.  $\text{HN}_2$  has been particularly valuable in the terminal cases of Hodgkin's disease, i.e., in individuals completely disabled by their disease, having visceral involvement, and running an irregular or relapsing fever. The use of a single course of  $\text{HN}_2$  in such cases has often resulted in a termination of the febrile state and its associated symptom, the drenching night sweat. Frequently, there is a dramatic upsurge in vitality and a resumption of normal or almost normal activity. Severe itching of the skin, with its common accompaniments of excoriations and ulcerations due to scratching has usually yielded to  $\text{HN}_2$  when previous x-ray therapy has proved completely ineffective.

In those cases that have become refractory to x-ray therapy, whether the Hodgkin's process is generalized or of a more or less localized character, the use of  $\text{HN}_2$  may be invaluable. A single therapeutic course of  $\text{HN}_2$  often results in a very rapid and striking response with a marked simultaneous reduction in large lymphoid masses and spleen and in amelioration of both local and constitutional symptoms. The pain of peripheral nerve or spinal cord involvement has yielded quickly in all our cases to  $\text{HN}_2$  therapy even though previous x-ray therapy has been completely ineffective. A frequent finding is the enhancement of sensitivity to x-ray therapy following a course of  $\text{HN}_2$ .

Thus,  $\text{HN}_2$  has proved invaluable in salvaging some of the apparently hopeless cases of Hodgkin's disease and in increasing their life span by periods ranging from two months to more than two years. Although the results in terminal cases have at times seemed almost *miraculous*, they have nevertheless been of temporary nature. Treatment with  $\text{HN}_2$  should in no sense be considered as curative.

The new sense of well being obtained with  $\text{HN}_2$  has however proved of great psychologic value and has often given the patient a renewed determination to cope with his illness.

Our experience with the treatment by  $\text{HN}_2$  of early or only slightly advanced cases has been too limited to warrant any definite therapeutic evaluation. In such cases, in which only a single group of nodes is apparently involved, the distinct possibility is present that some of the abnormal cells of the disease have already progressed beyond the local lesion. Although a sufficiently high dosage of x-ray therapy is usually productive of a sustained remission, the use of  $\text{HN}_2$  under these circumstances may help to destroy abnormal cells at a distance from the local process. Particularly in the early cases of Hodgkin's disease, drastic therapy by all available means is important. This may include radical extirpation of a mass of glands, heavy dosage of high voltage x-ray, and  $\text{HN}_2$ .

There seem to be few contraindications to the use of  $\text{HN}_2$ . In the presence of leukopenia, the granulocytes should be carefully watched and treatment with penicillin given when a distinct granulocytopenia develops. When jaundice is present  $\text{HN}_2$  should be given with particular care since further injury to the liver may develop. If anemia is present, transfusions should be given either prior to the course of  $\text{HN}_2$  or during its administration.

We have had better results in our cases with the use of smaller rather than larger doses of  $\text{HN}_2$ . A complete remission is usually attended with the use of doses smaller than the customarily recommended amount of 0.1 mg per Kg of body weight. Reactions, particularly those of a hematologic nature, are usually slight. Larger doses of  $\text{HN}_2$  may be productive of extremely severe and indeed irreversible reactions.

Although x-ray therapy is still the method of choice in the early cases of Hodgkin's disease and is productive of longer remissions than is  $\text{HN}_2$ , the combined use of x-ray and  $\text{HN}_2$  may prove to be better than that of either therapeutic method given alone. We have obtained the impression that  $\text{HN}_2$  is more specific against reticulo-endothelial cellular proliferations than against those of any other cell type.  $\text{HN}_2$  must therefore be considered as a definite addition to our present therapeutic armamentarium of attack against Hodgkin's disease, which we consider to be a form of reticulum cell proliferation. It is realized that the use of  $\text{HN}_2$  leaves much to be desired, since it destroys abnormal cells leaving others which continue to maintain neoplastic potentialities. These ultimately proliferate, leading to relapse. However, it is hoped that further research will result in the development of even more potent chemotherapeutic agents for the ultimate control of the disease.

#### SUMMARY

1. Methyl bis (B chloroethyl) amine ( $\text{HN}_2$ ) was given by intravenous route for the treatment of 50 successive cases of Hodgkin's disease, most of them severe and far advanced. Doses somewhat smaller than the usually recommended amount of 0.1 mg per Kg were used in courses of four to six injections.

2. Nausea and vomiting followed administration of the drug in 93.2 per cent

of cases. Chills and fever occurred in 12.4 and 6.8 per cent of cases respectively. Dyspnea, cyanosis and diarrhea were rare.

3. In previously untreated cases, remissions were of much shorter duration than those obtained with Roentgen therapy. However, striking remissions were commonly obtained in x-ray resistant cases. Remissions lasted from 17 to 331 days and in individuals receiving multiple courses were roughly proportional to the total dosage administered. A moderate prolongation of the remission period was obtained when HN<sub>2</sub> was combined with roentgen therapy.

4. Constitutional symptoms such as fever, night sweats, weakness and itching responded exceedingly well in most cases to HN<sub>2</sub> therapy. Many previously incapacitated patients were completely rehabilitated for several weeks to several months after a single course of HN<sub>2</sub> therapy.

5. Adenopathy and splenomegaly regressed in 70.2 and 71.7 per cent of cases respectively. Lymphoid masses previously resistant to x-ray therapy appeared to develop increased sensitivity to x-rays after a course of HN<sub>2</sub> therapy.

6. Patients with extensive mediastinal involvement and obstructive symptoms responded only moderately well while those with lesser degrees of involvement showed a better response.

7. Paraplegia due to intraspinal involvement was partially relieved in half the cases while pain due to similar involvement was dramatically relieved in all cases. Pain due to pressure upon peripheral nerves was similarly relieved in all cases.

8. A slight but definite fall in the erythrocyte and hemoglobin levels occurred within five to six days after the institution of therapy. Reticulocytes were maximally depressed on the sixth to tenth days. Of the leukocytic elements, the granulocytes were predominately affected, with maximal cytopenic levels on the twenty-first to twenty-fifth day. The leukocytes gradually returned to normal by the thirty-sixth to fortieth day. Cases presenting an initial leukopenia tended to develop normal leukocyte counts after an initial drop to low levels. The platelet count was affected in only 20.5 per cent of cases. Terminal cases at times developed marked pancytopenia.

9. In one case severe hemorrhagic complications due chiefly to thrombocytopenia followed the administration of the tris form of nitrogen mustard and gradually subsided after a very stormy course.

10. Progressive but temporary marrow hypoplasia followed nitrogen mustard therapy in eleven cases studied with serial marrow punctures. Erythroblastic depression was noted within twenty-four hours and granulocytic depression within forty-eight to seventy-two hours. The megakaryocytes proved to be the most resistant of the marrow elements. The marrow picture usually returned to normal spontaneously within a period of six to eight weeks after the cessation of therapy.

11. Lymph node punctures revealed degeneration and pyknosis of lymphocytes within twenty-four hours after the institution of therapy with a subsequent gradual disappearance of polymorphonuclear neutrophils, eosinophiles, plasma cells, reticulum cells and Dorothy Reed cells. Military foci of necrosis were demonstrated in a gland obtained at post mortem seven days after the institution of HN<sub>2</sub> therapy.

12. Military foci of necrosis were demonstrated in the liver of 3 cases dying be-

tween nine and nineteen days after the institution of  $\text{HN}_2$  therapy. No such findings could be found in a case in which death occurred fifty-four days after the initiation of therapy.

13. The therapeutic results with  $\text{HN}_2$  in Hodgkin's disease appeared to have little relationship to the histologic appearance of the involved tissue. The immediate response in so called Hodgkin's sarcoma was particularly striking, and in one case, a remission lasting about a year took place.

### CONCLUSIONS

1. Nitrogen mustard ( $\text{HN}_2$ ) is a useful drug in the treatment of Hodgkin's disease, particularly in severe cases with marked constitutional symptoms and visceral involvement. In these cases, a period of complete rehabilitation and a definite increase in life span of from two months to two years may follow the use of one or several courses of  $\text{HN}_2$ .

2.  $\text{HN}_2$  appears to have an almost specific affinity for the abnormal tissues of Hodgkin's disease. Although a chemical without any radioactivity, its effects resemble closely those of x-ray. It is however often effective in producing complete remissions in cases that have proved completely refractory to continued x-ray therapy. A resumption in radiosensitivity may follow the use of a course of  $\text{HN}_2$  therapy.

3.  $\text{HN}_2$  offers certain advantages other than simplicity of administration over x-ray therapy. Its quick action by intravenous route often results in a simultaneous reduction of all affected lymphoid tissues. In involvement of the spinal cord or peripheral nerves,  $\text{HN}_2$  is far more effective, particularly in pain relief, than is x-ray.  $\text{HN}_2$  is likewise more effective in bringing about relief of fever and severe generalized itching than is x-ray. The one outstanding characteristic of the drug is its effectiveness in inducing complete or partial remissions in certain generalized or febrile cases that have been completely unaffected by persistent x-ray therapy. Repeated remissions may be induced by giving repeated courses of  $\text{HN}_2$ .

4. In relatively early cases of Hodgkin's disease, x-ray therapy is the treatment of choice, primarily because longer remissions can be obtained than with  $\text{HN}_2$ . However, it is possible that the best form of therapy, even in these cases, is that of the combined use of  $\text{HN}_2$  and x-ray, the  $\text{HN}_2$  being given for its effect upon proliferating cells which may either be at a distance from the local lesion or else so situated as to remain untouched by x-ray.

5. With cautious use of the drug, the reactions following  $\text{HN}_2$  therapy are rarely severe enough to militate against its use. Severe granulocytopenia can be handled prophylactically by the use of penicillin. Severe thrombocytopenia rarely occurs. The only definite contraindication to the use of  $\text{HN}_2$  is the presence of jaundice, indicating some degree of hepatic dysfunction.

6. Doses of  $\text{HN}_2$  somewhat smaller than the generally recommended one of 0.1 mg per kg of body weight are usually completely effective and are furthermore productive of minimal reactions.

7. As with all very quickly acting and potent drugs,  $\text{HN}_2$  must be used with



great care. Properly used, it has a well defined place in the treatment of Hodgkin's disease. Although cures are not to be expected and remissions are temporary, such remissions offer great comfort to the patient seriously ill with Hodgkin's disease. It is possible that  $\text{HN}_2$  may be the forerunner of other even more effective chemotherapeutic agents.

## REFERENCES

- <sup>1</sup> MARSHALL E K. Mustard gas. *J A M A* 73 684 1919
- <sup>2</sup> MANDEL, M AND GIBSON W S. Clinical manifestations and treatment of gas poisoning. *J A M A* 59 1970 1917
- <sup>3</sup> PAPPENHEIMER A M, AND VANCE, M. The effect of intravenous injections of dichloroethyl sulfide in rabbits with special reference to its leucotoxic action. *J Exp Med* 31 71 1921
- <sup>4</sup> WARTHIN A S AND WELLER C V. The lesions of the respiratory and gastrointestinal tracts produced by mustard gas (dichloroethyl sulphide). *J Lab & Clin Med* 4 229 1919
- <sup>5</sup> LYNCH V, SMITH, H W AND MARSHALL, E K. On dichloroethyl sulphide. *J Pharm & Exper Therap* 12 265, 1919
- <sup>6</sup> KRUMBHAAR E B AND KRUMBHAAR H D. The blood and bone marrow in yellow cross gas (mustard gas) poisoning. *J Med Res* 40 497 1919
- <sup>7</sup> GILMAN A, AND PHILIPS F S. The biologic actions and therapeutic applications of the B chloroethyl amines and sulfides. *Science* 103 409 1946
- <sup>8</sup> JACOBSON, L C. Nitrogen mustard therapy. *J A M A* 132 263 1946
- <sup>9</sup> GOODMAN L S WINTROBE M M DAMESHEK W GOODMAN M J GILMAN A AND MCLENIGAN M T. Nitrogen mustard therapy. *J A M A* 132 126 1946
- <sup>10</sup> WINTROBE M M AND HUOUELY C M. Nitrogen mustard therapy for Hodgkin's disease lymphosarcoma the leukemias and other disorders. *Cancer* 3 357, 1948
- <sup>11</sup> CRAVER L F. Recent advances in the treatment of lymphomas leukemia and allied disorders. *Bull New York Acad Med* 24 3 1947
- <sup>12</sup> ZANES R P JR DOAN C A AND HOSTER H A. Studies in Hodgkin's syndrome VII. Nitrogen mustard therapy. *J Lab & Clin Med* 33 1002 1948
- <sup>13</sup> AP THOMAS M I R AND COLLUMBINE H. Nitrogen mustard in Hodgkin's disease. *Lancet* 1 899 1947
- <sup>14</sup> ALPERT L K AND PETERSON S S. The use of nitrogen mustard in the treatment of lymphomas. *Bull U S Army Med Dept* 7 187 1947
- <sup>15</sup> TALBOTT J H. Clinical manifestations of Hodgkin's disease. *New York State J Med* 47 1883 1947
- <sup>16</sup> HETTIO R A. Results of methyl bis (beta chloroethyl) amine hydrochloride therapy. *J Lab & Clin Med* 32 1431 1947
- <sup>17</sup> SHERRY M. Nitrogens in the treatment of Hodgkin's disease and lymphosarcoma. *South M J* 41 118 1948
- <sup>18</sup> WILKINSON J F AND FLETCHER F. Effect of B chloroethylamine hydrochlorides in leukemia Hodgkin's disease and polycythemia vera. *Lancet* 2 540 1947
- <sup>19</sup> TAFFEL M. Experiments in the treatment of neoplastic disease with nitrogen mustard. *Yale J Biol & Med* 19 971 1947
- <sup>20</sup> HENSTELL H H TOBER J N AND NEWMAN B A. The influence of nitrogen mustard on mycosis fungoides. *Blood* 2 564 1947
- <sup>21</sup> PHILPOTT O S WOODBURN A R AND WALDRIFT G A. Nitrogen mustard in the treatment of mycosis fungoides. *J A M A* 135 631 1947
- <sup>22</sup> OSBORNE E D JORDON J W HOAK F C AND PSCHIRER F J. Nitrogen mustard therapy in cutaneous blastomatous disease. *J A M A* 135 1123 1947
- <sup>23</sup> KIERLAND R R WATKINS C H AND SHULLENBERGER C C. The use of nitrogen mustard in the treatment of mycosis fungoides. *J Int Derm* 9 195 1947
- <sup>24</sup> SMIBER, G E. The treatment of Boeck's sarcoid with nitrogen mustard. *South M J* 41 11 1948
- <sup>25</sup> KARNOFKY, D A ET AL. A tentative analysis of results obtained with nitrogen mustard in the treatment of carcinoma of the lung. Unpublished data

- <sup>16</sup> — A tentative analysis of results obtained with nitrogen mustard in miscellaneous types of neoplastic disease Unpublished data
- <sup>17</sup> — Chemotherapy of neoplastic disease III Agents of clinical value *New England J M* 239 299 1948
- <sup>18</sup> ANSLOW, W P KARNOFSKY D A, VAL JAGER, B, AND SMITH H W The toxicity and pharmacological action of the nitrogen mustards and certain related compounds *J Pharm & Exp Therapy* 97 224 1947
- <sup>19</sup> KARNOFSKY D A GRAEF I, AND SMITH, H W Studies on the mechanism of action of nitrogen and sulfur mustard in vivo *Am J Path* 24 275, 1948
- <sup>20</sup> BOURSNELL, J C, COHEN, J A DIXON M, FRANCIS, G E OREVILLE G D NEROHAM D M, AND WORMALL A The fate of injected mustard gas (containing radio-active sulfur) in the animal body *Biochem J* 40 756 1946
- <sup>21</sup> FEINDENWALD, J S, AND BUSCHKE W Studies on the physiology biochemistry and cytopathology of the cornea in relation to the injury by mustard gas and allied toxic agents V Nuclear fragmentation produced by mustard and nitrogen mustard in the corneal epithelium *Bull Johns Hopkins Hospital* 22 161 1948
- <sup>22</sup> DAMESHEK W A method for the simultaneous enumeration of blood platelets and reticulocytes *Arch Int Med* 50 579 1932
- <sup>23</sup> SPURR C L JACOBSON L O SMITH T R AND BARRON E S G The clinical application of a nitrogen mustard compound methyl bis (B chloroethyl) amine to the treatment of neoplastic disorders of the hemopoietic system *Cancer Research* 7 51, 1947
- <sup>24</sup> JACKSON H AND PARKER, F Hodgkin's disease *New England J M* 230 1 1944
- <sup>25</sup> WEIL, A Spinal cord changes in lymphogranulomatosis *Arch Neurol & Psychiat* 26 1009, 1931
- <sup>26</sup> BOURSNELL, J C, FRANCIS G E AND WORMALL, A. The immunologic properties of proteins treated with mustard gas and some related compounds *Biochem J* 40 768 1946
- <sup>27</sup> GRAEF O KARNOFSKY D A VAL JAGER, B KRICHESKY, B AND SMITH H W The clinical and pathologic effects of the nitrogen and sulfur mustards in laboratory animals *Am J Path* 24 1 1948
- <sup>28</sup> ROSENTHAL E Nitrogen mustard therapy combined with splenectomy *Lancet* 1 408, 1948
- <sup>29</sup> URTEAGA O B Tratamiento de las Leucemias con Alkilaminas Peru, 1947
- <sup>30</sup> BLOOM M A AND BLOOM W A The radiosensitivity of erythroblasts *J Lab & Clin Med* 32 654 1948
- <sup>31</sup> HUNDRED J E Histologic changes occurring in the hemopoietic organs of albino rats after single injections of two chloroethyl vesicants *Arch Path* 43 253 1947
- <sup>32</sup> CAMERON G R COURTICE F C, AND JONES R P The effects of B B' dichloroethyl methylamine hydrochloride on the blood forming tissues *J Path & Bact* 59 425 1947
- <sup>33</sup> BLOCK M SPURR C L JACOBSON L O AND SMITH T R Histopathologic effects of nitrogen mustard therapy upon normal and neoplastic hematopoietic tissues *Am J Clin Path* 28 671 1948
- <sup>34</sup> SPITZ, S The histological effects of nitrogen mustards on human tumors and tissues *Cancer* 1 383, 1948
- <sup>35</sup> ZIMMERMAN H Quoted in reference 37
- <sup>36</sup> BARRON E S G, BARTLETT G R MILLER Z B MEYER J AND SEEGMILLER J E The effect of nitrogen mustards on enzymes and tissue metabolism I The effect on tissue metabolism *J Exp Med* 27 503 1948

## PREPARATION OF STABILIZED SOLUTIONS OF HEMOGLOBIN

By ROBERT B PENNELL, PH D, AND WILLIAM ELLIOTT SMITH, B S

**D**URING the recent war the need for whole blood for its oxygen-carrying capacity and the supply of human red cells derived from the plasma program revived interest<sup>1-3</sup> in the long controversy as to the usefulness of hemoglobin solutions in therapy. That there should have been such a controversy was due in large part to the lack of availability for study of a standard hemoglobin solution of high stability prepared with adequate bacteriologic control, and to the consequent difficulty of interpreting much of the published work. This has been emphasized by Hamilton, et al.<sup>1</sup> We had the privilege of working with two of the groups which undertook the reinvestigation of this problem, that of Dr. William R. Amberson of the University of Maryland Medical School and that of Dr. Donald D. Van Slyke of the Hospital of the Rockefeller Institute, N. Y. From the work of each of these groups a standard hemoglobin solution suitable for clinical study was developed.<sup>1, 2, 4, 29</sup> Stability of these solutions could be maintained, however, only by special treatment, exhaustion of oxygen by high vacuum in the one instance and refrigeration in the other.

Hemoglobin in solution exists in three forms<sup>30</sup> which are in equilibrium, two of which, oxyhemoglobin and reduced hemoglobin, are physiologically active (i. e., they can act as oxygen carriers) and one of which, methemoglobin, is physiologically inactive. One of the physiologically active forms, reduced hemoglobin, is stable<sup>31-34</sup> and the other is not.<sup>1</sup> The instability of hemoglobin in solution is first manifested by its conversion to the physiologically inactive methemoglobin.

The three forms of hemoglobin also occur within the red cell, which is so constructed, however, that the hemoglobin within it is maintained in an active oxygen-carrying form, the inactive methemoglobin being kept at an extremely low level.<sup>35-37</sup> In the course of our studies we found methods of preparing solutions of hemoglobin by which the mechanism for maintenance of its oxygen-carrying capacity was preserved. This resulted in a hemoglobin solution which would convert itself to the stable reduced hemoglobin form,<sup>34</sup> thus obviating the special treatments usually necessary for storage of the solutions. The present study is concerned with delineation and demonstration of the factors of importance to the preparation of this type of solution.

### METHODS, TESTS AND EQUIPMENT

Total hemoglobin and methemoglobin contents of hemoglobin solutions were determined with the Evelyn photoelectric colorimeter employing the method of Evelyn and Malloy.<sup>38</sup> The values so obtained were found to check well with those obtained in other laboratories by other methods.<sup>39</sup>

The pH of solutions was determined by glass electrode without dilution of the hemoglobin solutions. Sodium and potassium concentrations of solutions were determined by the flame photometer.<sup>40</sup>

Tests for pyrogenic substances were carried out in accordance with the Minimum Requirements for Pyrogen Tests on Biologic Products from Blood Serum. Nov. 19, 1945. National Institute of Health.

From the Department of Immunoochemistry, Sharp and Dohme, Inc., Glenolden, Pennsylvania.

Sterility tests and animal safety tests were carried out in accordance with the Federal Register of September 16, 1947 as amended in January 1948. Since hemoglobin solutions form precipitates when added to the culture medium used in the sterility test, subcultures were made at the end of one week and the final test was read two weeks after the date of testing.

Pyrogen free water was prepared by double distillation, followed by immediate use, or by immediate storage at 2°C for not more than sixteen hours.

Total reducing substances and nonfermentable reducing substances were determined by the method of Benedict.<sup>48</sup>

Inorganic phosphorus was determined by the method of Embden and Fetter.<sup>49, 50</sup>

Lipid phosphorus was determined by the method outlined in *Quantitative Clinical Chemistry*, by Peters and Van Slyke, vol. II, page 884, 1st Edition, 1932.

## PREPARATION OF HEMOGLOBIN SOLUTIONS

*Source of Blood Cells* Sterile human red blood cell residues were obtained from commercial bleedings from the plasma processing unit of Sharp and Dohme. The bleedings were drawn in sodium citrate solution and approximately seventy-two hours elapsed between the time of bleeding and the time that the red cell residues were available for hemoglobin preparation.

*General Measures Observed* Rapidity of operation and maintenance of optimal working conditions were employed rather than aseptic handling during preparation of the solutions. Starting with sterile red cells, the entire operation was invariably completed and the final solutions sterilized within the course of eight hours. All work was performed at 2°C in a laboratory equipped with Sterilamps. All equipment coming in contact with the solutions was carefully cleaned and rinsed with pyrogen free water immediately before use. Pyrogen free water was used throughout for all dilutions and all solutions added to, or coming in contact with, the cells and hemoglobin. All hemoglobin solutions were submitted for sterility and for pyrogen and safety testing immediately after preparation.

*Washing and Laking of Red Blood Cells* From 2 to 5 liters of packed human red cells were washed by suspension in 2 volumes of 6 per cent dextrose solution containing 0.15 per cent nicotinic acid amide and 0.006 per cent ammonia, followed by centrifugation in a laboratory model Sharples Super Centrifuge using the Sharples blood separator bowl. With this bowl the wash solution is delivered from one outlet and the washed cells from another. The cells were ruptured during centrifugation and were caught in a container holding a small amount of nicotinic acid amide solution (containing sufficient nicotinic acid amide to provide 0.15 per cent in the estimated final volume of solution). The washed, ruptured cells were diluted with 2 volumes of dextrose solution (sufficient dextrose is used to provide 6 per cent in the estimated final volume of hemoglobin solution).

*Precipitation of Stroma*<sup>1</sup> The mixture was adjusted to pH 5.7-5.8 with 0.1 N hydrochloric acid at which pH the stroma was readily removed by centrifugation in a large Sharples Super Centrifuge using a clarifying bowl. The addition of the acid was accompanied by brisk mechanical stirring. The acid was allowed to run in a thin stream from a capillary pipette near the vortex of the stirring solution. Approximately 250 cc of 0.1 N hydrochloric acid per liter of solution may be added before determining the pH.

*Removal of Excess Potassium*<sup>1</sup> The centrifuged mixture was treated with sodium zeolite (decalso)\* to reduce the potassium content. Approximately 30 Gm of sodium zeolite per liter of solution was added and the mixture was stirred gently for ½ hour, after which the sodium zeolite was allowed to settle for ten to fifteen minutes. The solution was then decanted.

*Adjustments to the Final pH and Composition* Sufficient solid sodium bicarbonate was added to the solution to neutralize the hydrochloric acid added and to provide a slight excess.<sup>1</sup> It was found that 7.9 Gm of sodium bicarbonate per liter of solution provided a pH of 7.2 to 7.3 at this point. The final concentration of hemoglobin was adjusted to approximately 7 per cent, that of dextrose to 6 per cent and that of nicotinic acid amide to 0.15 per cent. To the solution was added, per liter, 5 cc of ammonium hydroxide solution (5 parts of Baker's A.C.S. ammonium hydroxide to 100 parts of water) to provide a concentration of 0.006 per cent  $\text{NH}_3$ , 4 cc of 1 per cent merthiolate (to provide a concentration of 1:25,000), 24 mg

\* Manufactured by the Permutit Co., New York, N. Y. Since earlier work had indicated the occasional presence of pyrogenic material in decalco it was always washed as described by Smith and Pennell in *J. Biol. Med.* 54, 715, 1947.

$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (1 cc of 2 per cent solution) 23 mg  $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$  (1.15 cc of 2 per cent solution) and 19.5 mg  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$  (0.99 cc of 2 per cent solution) providing 0.1 millimolar concentration of each of the metals, and 20 mg of Nile blue (2 cc of 1 per cent solution). In a few of the later solutions 1 Gm per liter of the calcium salt of hexose diphosphate\* was added before stirring with decalzo. The  $\text{Ca}^{++}$  ions were removed from solution by the ion exchange agent.

**Sterilization by Sinter Filtration** The solution was filtered through K6 clarifying pads and sterilized by filtration through S4† pads using a Republic filter press. The clear red filtrate was caught in a sterile bottle containing a sterile syphon which could be used for filling the solution into a series of small containers. Samples taken at the time of filling these small containers were tested for pyrogenicity, sterility and safety. The solutions were held at 2 C until the completion of these tests.

### PROPERTIES OF HEMOGLOBIN SOLUTIONS

Solutions prepared according to the procedure just described were crystal clear and of deep red color. The hemoglobin of the solutions was more than 98 per cent active. Thorough examination indicated complete inability of the solutions to agglutinate A, B or O cells. Examination of the serum of patients before and two weeks after the injection of these solutions has shown no increase in the titer of anti-A and anti-B isohemagglutinins. Lipid phosphorus determinations showed that upon removal of the stroma less than 10 per cent of the lipid phosphorus remained in the solution, a figure corresponding closely to the 5-10% of lipid carbon reported by Hamilton, et al.<sup>1</sup> In a typical preparation the solution contained 5.56 milliequivalents of potassium, as compared to 19 milliequivalents before decalzo treatment. These solutions, when in sealed containers with little air space were completely converted to reduced hemoglobin in one to two days at 37 C, in two to three days at 25-27 C, or in seven to eight weeks at 2 C. During this conversion the presence of methemoglobin in quantities greater than 2-3 per cent of the total pigment was not detectable by daily examination. The reduced hemoglobin so obtained has been observed for twenty four months at 25-27 C without a change in the percentage of active hemoglobin. There may be, however, gradual deposition of a sediment during this time, the amount of sediment being a function of the speed of disappearance of oxygen, the amount of oxygen to be consumed, i.e., the amount of air space in the bottle, and the temperature of storage. No noticeable sediment has been encountered in solutions stored at 2 C. (Cursory examination of the sediment has shown it to be in part carbohydrate in nature, giving no reduction before hydrolysis and indicating a mixture of aldo- and keto-sugars after hydrolysis.) Shaking of the solutions, with consequent foam formation, may result in the appearance of films due to surface denatured protein. None of these phenomena has been found to have influence, detectable by the methods employed on the activity of the hemoglobin itself. These solutions lend themselves readily to lyophilization as will be described in a subsequent publication.

### EFFECTS OF VARIOUS STEPS ON THE CAPACITY OF THE SOLUTIONS TO FORM REDUCED HEMOGLOBIN

Neill<sup>24</sup> showed that since oxyhemoglobin and reduced hemoglobin have different colors, hemoglobin solutions in sealed containers could act as indicators of the

\* Schwartz Laboratory Inc., New York N. Y.

† Republic Filter Corp. Paterson N. J.

loss of oxygen from solution due to the action of enzyme systems which he added Warburg<sup>61</sup> had similarly made use of this phenomenon in following respiration of intact avian red cells Neill showed that when removal of oxygen from solution was rapid, the accumulation of methemoglobin did not occur It has since been shown by many workers<sup>42-50</sup> that methemoglobin itself may be reconverted to active hemoglobin by the action of enzyme systems Evelyn and Malloy<sup>65</sup> developed methods based on the light absorption of the cyan derivatives of these pigments which allow this interconversion to be followed quantitatively The aging data reported below were obtained by measurements of the accumulation and disappearance of methemoglobin by the method of Evelyn and Malloy in sterile hemoglobin solutions stored in sealed vials with a small amount of air space A separate vial was opened for each determination and was then discarded The aging test

TABLE 1—*Changes in Reducing Substances and Inorganic Phosphorus During Preparation of Hemoglobin Solutions*

I. Cell residues obtained immediately after bleeding II Cell residues obtained from the plasma unit (approx seventy two hours after bleeding) III Washed cells IV Hemoglobin solution after removal of stroma V Hemoglobin solution after treatment with decalson VI Hemoglobin solution after filtration No dextrose was added at any stage of this study

	Mg per gram of Nitrogen		
	Total reducing substances	Nonfermentable reducing substances	Inorganic P
I	13.4	12.5	0.98
II	2.6	0.90	4.4
III	1.7	1.28	2.55
IV	1.7	0.81	3.05
V	1.5	0.48	2.62
VI	1.42	0.42	2.11

for a particular solution was considered to be completed when the accumulated methemoglobin had been reconverted to hemoglobin, or, when methemoglobin accumulation was not detected, upon the appearance of the typical grape-juice color of reduced hemoglobin

The data to be presented are strictly comparable only when obtained from a single pool of cells In table 1 it is evident that the amount of fermentable reducing substances in the cells obtained from the plasma laboratory was much less than in cells from a fresh bleeding, and at the same time, the inorganic phosphorus was elevated It is well known that after exhaustion of the available substrate, red cells cannot be restored to their original state of metabolism<sup>6</sup> The status of the red cells to be used for preparation of the particular type of hemoglobin solution under discussion will, then, be very important in its effect on the properties of the solution Uncertainty as to the exact state at which a given batch of cells might be, necessitated the finer comparisons being made only from a single lot of cells

In the solutions to be described below, with the exception of that of figure 1, the procedure of preparation was as described above, with only the variations noted in each case None of the solutions with the exception of that of figure 8 contained hexose diphosphate

Figure 1 gives the aging at room temperature of a hemoglobin solution made by the method of Hamilton et al.<sup>1</sup> Methemoglobin accumulated steadily. Such solutions examined after one year at  $-10^{\circ}\text{C}$  showed no accumulation of methemoglobin over that of the original solution. After two years of storage at  $-10^{\circ}\text{C}$ , however, such solutions have from 30 to 50 per cent of their total pigment in the form of methemoglobin.

Figure 2 gives aging data of a typical solution prepared by the methods just described. In the lower curve the hemoglobin was completely reduced on the

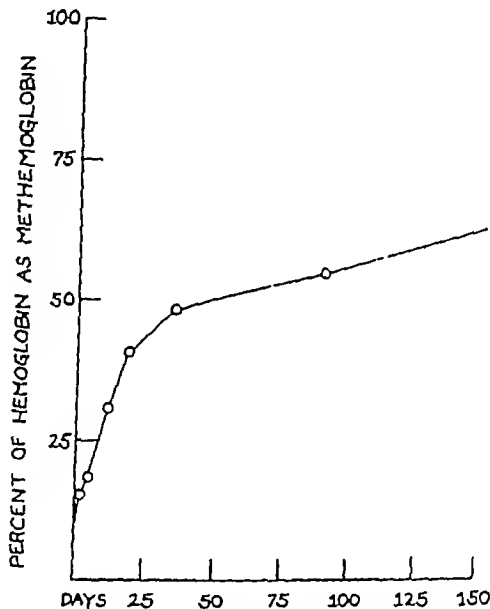


FIG. 1—AGING OF A HEMOGLOBIN SOLUTION PREPARED BY THE METHOD OF HAMILTON ET AL.<sup>1</sup> AT ROOM TEMPERATURE

seventh day. At the time this particular study was made, the methemoglobin content was being determined at weekly intervals. Later studies with daily determinations have revealed no deviation from this curve. The upper curve demonstrates that during storage there was no loss in total pigment content. Examination of these solutions for the presence of reducing substances following conversion of the pigment to reduced hemoglobin has revealed a drop in reducing substances from the original 6 per cent to 0.3 per cent.

Figure 3 illustrates that when dextrose was used, both in washing the cells and in the final solution, but neither nicotinic acid amide nor ammonia were used, methemoglobin gradually accumulated and then disappeared with reconversion to reduced hemoglobin. If no dextrose was used, but both nicotinic acid amide and ammonia were used there was no reconversion of methemoglobin. If the cells were

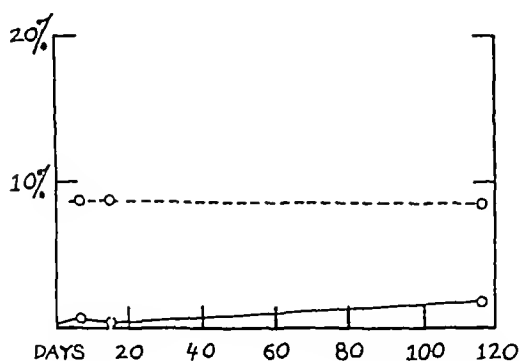


FIG 2—AGING AT ROOM TEMPERATURE OF A HEMOGLOBIN SOLUTION PREPARED BY THE METHODS DESCRIBED ABOVE

— Per cent of hemoglobin appearing as methemoglobin  
 --- Grams per cent of total hemoglobin in the solution

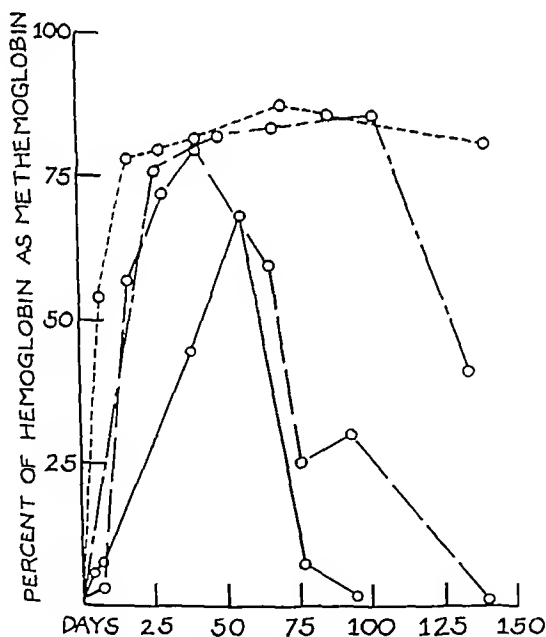


FIG 3—EFFECT OF DEXTROSE ON THE AGING OF HEMOGLOBIN SOLUTIONS AT ROOM TEMPERATURE

— Aging data for a hemoglobin solution in the preparation of which dextrose alone was used and from which the stroma was removed without adjustment of pH  
 --- Cells washed in 1 per cent sodium chloride solution but the solution made up as usual  
 ..... Cells washed as usual but no dextrose added to the solution after washing  
 -.-.- No dextrose added at any stage of preparation



washed in salt solution but the final solution contained dextrose reconversion of methemoglobin was again seen. If the cells were washed with 6 per cent dextrose solution but the solution was made up in physiological salt solution reconversion was slow and partial. These data indicated to us that the presence of dextrose in the final solution was essential, and its presence in the wash water desirable. It is interesting to note that maintenance of the cells and the hemoglobin con-

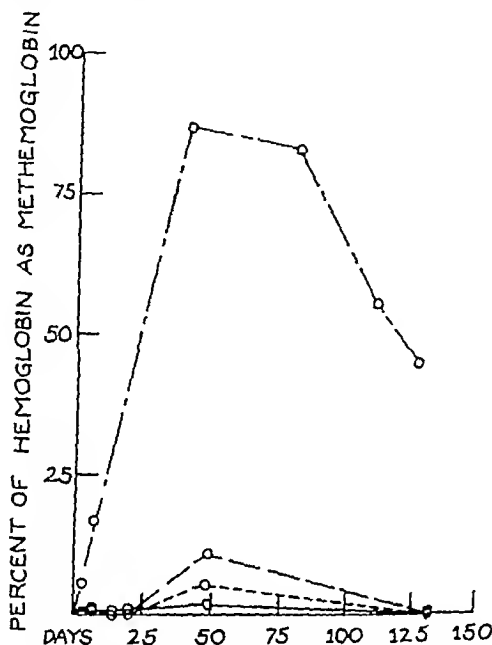


FIG. 4.—EFFECT OF NICOTINIC ACID AMIDE ON AGING OF HEMOGLOBIN SOLUTIONS AT ROOM TEMPERATURE

— Aging data for a solution made without addition of nicotinic acid amide at any stage  
 - - - - - 0.075 per cent nicotinic acid amide in final solution  
 - - - - - 0.15 per cent nicotinic acid amide in final solution  
 ——— 0.3 per cent nicotinic acid amide in final solution

stantly in the presence of dextrose alone will bring about reconversion of the methemoglobin. Three lots of cells were used in these preparations.

Figure 4 shows the effect of nicotinic acid amide on reconversion of methemoglobin to reduced hemoglobin. The upper curve shows aging data from a solution made without nicotinic acid amide at any stage. Reconversion of the methemoglobin formed was slow and incomplete. The three curves at the bottom of this graph represent a single hemoglobin solution, divided into 3 portions to which 0.075 per cent, 0.15 per cent and 0.3 per cent nicotinic acid amide were added respectively. Although as seen from figure 3 dextrose is essential, the efficacy of nicotinic acid amide is self evident.

Figure 5 The solid curve represents aging data obtained with a hemoglobin solution in which the neutralization of hydrochloric acid and adjustment to pH 7.3 was achieved with ammonium hydroxide. Neutralization with sodium hydroxide and the addition of ammonia was also effective. The two higher curves represent neutralization with sodium hydroxide and potassium carbonate with no ammonia addition. While not essential, ammonia is obviously advantageous to reconversion

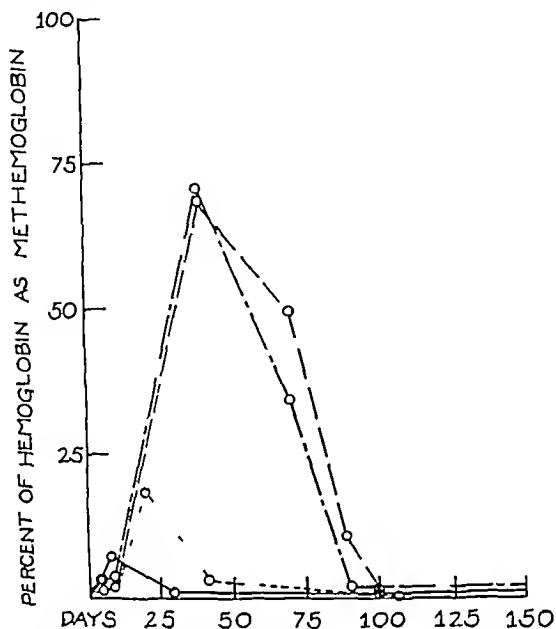


FIG 5—EFFECT OF AMMONIA ON THE AGING OF HEMOGLOBIN SOLUTIONS AT ROOM TEMPERATURE

- Hemoglobin solution adjusted from pH 5.8 to pH 7.3 with ammonium hydroxide
- - - Hemoglobin solution neutralized with sodium hydroxide and 0.006 per cent ammonia added
- · - · Hemoglobin solution neutralized with sodium hydroxide no ammonia added at any stage
- Hemoglobin solution neutralized with potassium carbonate no ammonia added at any stage

of methemoglobin to hemoglobin. Curves in which an attempt was made to evaluate the optimum amount of ammonia all coincided with the base line, and are not shown.

Figure 6 The use of an ion exchange agent made it seem likely that traces of metals essential to some of the enzyme systems might be removed.  $Mg^{++}$  and  $Mn^{++}$  ions are well known to be important to the action of some enzymes and it has been suggested<sup>11</sup> that  $Mg^{++}$ ,  $Mn^{++}$  and  $Co^{++}$  may have protective action against certain types of inhibition of enzyme systems. The central curve represents aging data obtained with a solution to which no metal ions were added. The dotted line represents another portion of the same solution to which  $Mg^{++}$ ,  $Mn^{++}$  and  $Co^{++}$  ions

were added. Reconversion was much quicker in this solution. To separate vials of the control solution, sterile solutions of manganese chloride alone, cobalt chloride alone and magnesium sulfate alone were injected. The aging data were similar for

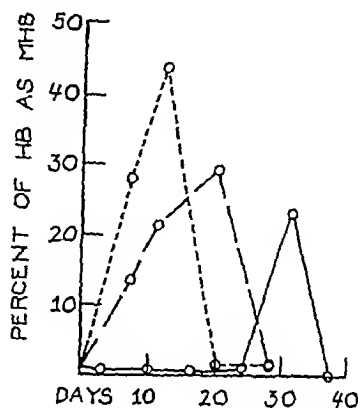


FIG 6—EFFECT OF  $Mg^{++}$ ,  $Mn^{++}$  AND  $Co^{++}$  IONS ON THE AGING OF HEMOGLOBIN SOLUTIONS AT ROOM TEMPERATURE.

- Hemoglobin solution containing no added  $Mg^{++}$ ,  $Mn^{++}$  or  $Co^{++}$  ions  
 ----- A portion of the same hemoglobin solution with 0.1 millimole of  $Mg^{++}$ ,  $Mn^{++}$  and  $Co^{++}$  added  
 -.-.-.- A portion of the same hemoglobin solution with 0.1 millimole of  $Co^{++}$  added

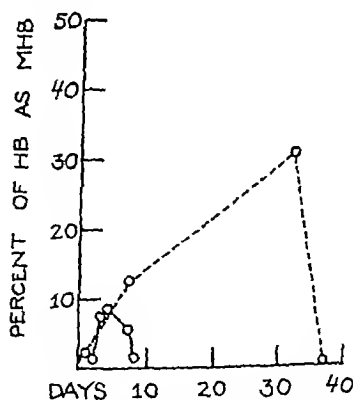


FIG 7—EFFECT OF NILE BLUE ON THE AGING OF HEMOGLOBIN SOLUTIONS AT ROOM TEMPERATURE

- Hemoglobin solution containing no nile blue  
 -.-.-.- A portion of the same hemoglobin solution containing 0.001 per cent nile blue

each set and are represented by the solid line curve. The long lag period preceding the appearance of methemoglobin was unexpected and its significance is not apparent to us.

Figure 7. A hemoglobin solution was made containing no nile blue. To one portion of this solution nile blue was added. The results are evident from this graph.

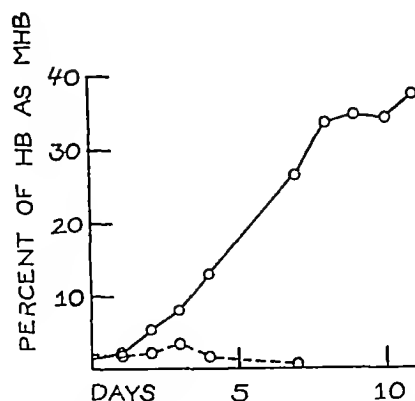


FIG 8—EFFECT OF HEXOSE DIPHOSPHATE ON AGING OF HEMOGLOBIN SOLUTIONS AT ROOM TEMPERATURE

— Hemoglobin solution containing no hexose diphosphate

----- A portion of the same solution containing 0.1 per cent of hexose diphosphate

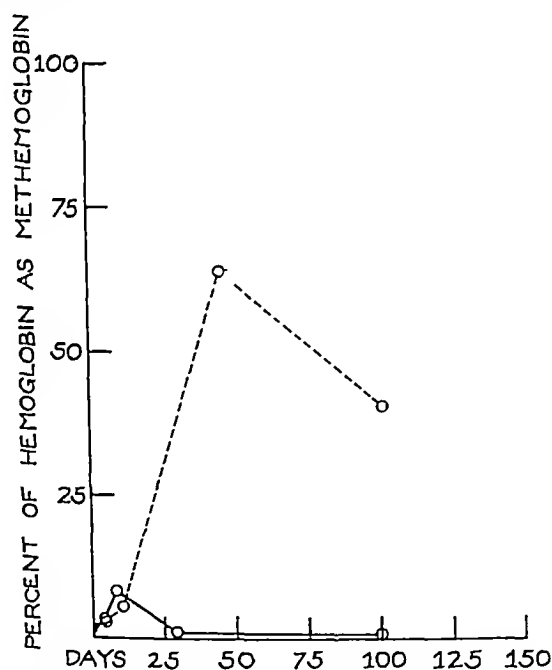


FIG 9—EFFECT OF FILTRATION ON AGING OF HEMOGLOBIN SOLUTIONS AT ROOM TEMPERATURE

— Filtered through an S-6 pad

----- A portion of the same solution filtered through an S-3 pad

The ratio solution filtered/pad surface area, was identical for the two filtrations

Figure 8 As noted above hexose diphosphate has been incorporated in some of the more recent hemoglobin solutions. This figure shows aging data obtained with a solution to one portion of which hexose diphosphate was added. This portion

demonstrated a somewhat more active reconversion of methemoglobin than did the portion not containing hexose diphosphate

Figure 9 demonstrates the importance of the type of filter pad used for sterilization of hemoglobin solutions. A hemoglobin solution was divided before sterilizing filtration, one portion being filtered through a Republic S6 pad and one portion being filtered through the finer meshed Republic S3 pad. The ratio of the volume of hemoglobin solution filtered to the pad surface was identical for each portion. It can readily be seen that the tighter pad removed something from the solution that was important for the reversion of methemoglobin to hemoglobin.

### DISCUSSION

It has been reported by all previous workers that the ability of the erythrocyte to utilize dextrose as a substrate is lost at the time of, or soon after, the disruption of the cell.<sup>4, 43, 48, 50, 53, 64, 68</sup> The present data suggest that these previous findings must be qualified, for if the cells are maintained in the presence of dextrose during hemolysis, the ability to utilize dextrose continues. It is true that when the only precaution taken is maintenance of the cells in the presence of dextrose the utilization of dextrose after removal of the stroma is extremely slow. When, however, ammonia, a known stimulant of respiration,<sup>69, 70</sup> and nicotinic acid amide, a known protector of enzymic action,<sup>71</sup> are also present, utilization of dextrose and consumption of oxygen are appreciably accelerated. In the presence of dextrose, nicotinic acid amide and ammonia the additional contributions of added metals, nile blue and hexose diphosphate to the speed of consumption of oxygen from the solutions is relatively slight. The data suggest that the success of the preparation of the type of hemoglobin solution under discussion is dependent on the maintenance of as high a state of metabolic activity as possible in the red cell during the preparation. This approach may well lead to the development of solutions of still higher activity. One definite limitation to the activity of such solutions is suggested by the past emphasis on the importance of the cell structure for the activity of the cellular enzymes.<sup>53, 64, 66, 68</sup> The data in figure 9 give indication that some of the structurally important elements are necessary to the highest activity and can be removed by further treatment.

The respiratory activity of the adult mammalian red cell is known to be small. It was first clearly demonstrated by the use of dyes of proper oxidation-reduction potential.<sup>65, 7, 75</sup> Methylene blue, the dye most studied in this connection, not only catalyzes the action of the cellular enzyme systems but also catalyzes the formation of methemoglobin from hemoglobin. Kiese<sup>48</sup> pointed out that nile blue acted as a catalyst for the enzyme systems but did not catalyze the other reaction. It was used in these studies for that reason.

Since the entire cell residues from plasma have been used in these studies, it cannot be stated with accuracy that the white cells do not contribute to the self-reduction noted. The work of Bird<sup>76</sup> suggests that their contribution would not be a major one.

Although the solutions under discussion readily utilize dextrose as a substrate, the fact that added hexose diphosphate further increases the rate of disappearance

of oxygen is of importance since aging studies have indicated that the appearance of the reduced solutions improves in inverse proportion to the time of reduction.

The presence of specific enzymes capable of bringing about the actions observed has not been proven in this study. The factors affecting the data reported are highly suggestive that this action is enzymic, however. It is hoped to pursue this study further at a later date. It is also hoped to find something of the nature of the by products formed.

### SUMMARY

It is possible to prepare self-stabilizing solutions of hemoglobin from human erythrocytes by the use of dextrose, nicotinic acid amide and ammonia during the preparation and in the final solutions themselves.  $\text{Co}^{++}$ ,  $\text{Mn}^{++}$  and  $\text{Mg}^{++}$  ions, Nile blue and hexose diphosphate contribute to the speed of stabilization of these solutions. Stabilization is obtained by the faculty of the solutions, presumably by enzymic action, to convert the hemoglobin to the reduced form and to maintain it in this form. The hemoglobin solutions described are suitable for intravenous administration.

### ACKNOWLEDGMENT

We wish to acknowledge our deep indebtedness to Miss Lois Priester and to Mr. Edward Smith for their technical assistance in this work. We wish also to express our gratitude to the Biological Production Division and to the Biological Control Division of Sharp and Dohme for assistance in many phases of the work.

### REFERENCES

- <sup>1</sup>HAMILTON P. B., FARR L. E., HILLER A. AND VAN SLYKE D. D. Preparation of hemoglobin solutions for intravenous infusion. *J. Exp. Med.* **86**: 455, 1947.
- <sup>2</sup>FARR L. E., HILLER A., AND VAN SLYKE D. D. Preparation of dried hemoglobin without loss of activity. *J. Exp. Med.* **86**: 465, 1947.
- <sup>3</sup>HAMILTON P. B., HILLER A. AND VAN SLYKE D. D. Renal effects of hemoglobin infusions in dogs in hemorrhagic shock. *J. Exp. Med.* **86**: 477, 1947.
- <sup>4</sup>ANDERSON W. R. Clinical experience with hemoglobin saline solutions. *Science* **106**: 117, 1947.
- <sup>5</sup>—, RHOODE C. M. AND JENNINGS J. E. Clinical experience with hemoglobin-saline solutions. *Fed. Proc.* **5**: 2, 1946.
- <sup>6</sup>RATHER L. J. Renal atrophy and intracellular digestion of intraperitoneally injected hemoglobin in rats. *J. Exp. Med.* **87**: 163, 1948.
- <sup>7</sup>MCCARTHY M. D., AND PARKINS W. M. Comparative effectiveness of albumin, globin, hemoglobin, gelatin, oxypolygelatin, saline, Ringer's blood and plasma upon the survival of rats subjected to standardized scald burns. *Am. J. Physiol.* **156**: 418, 1947.
- <sup>8</sup>LAUGH J. J. The influence of injections of homologous hemoglobin on the kidneys of normal and dehydrated animals. *J. Exp. Med.* **86**: 153, 1947.
- <sup>9</sup>HARRISON H. E., BUNTINO H., ORDWAY N. K. AND ALBRINK, W. S. The pathogenesis of the renal injury produced in the dog by hemoglobin or methemoglobin. *J. Exp. Med.* **86**: 339, 1947.
- <sup>10</sup>HAMILTON A. S., PARKINS W. M. AND WALZER F. A comparison of ten infusion fluids in the treatment of moderate and severe hemorrhage in animals. *Am. J. Physiol.* **150**: 641, 1947.
- <sup>11</sup>FLINK E. B. Blood transfusion studies: relationship of hemoglobinemia and of pH of urine to renal damage produced by injection of hemoglobin solutions into dogs. *J. Lab. & Clin. Med.* **32**: 223, 1947.
- <sup>12</sup>LITTMAN R. W. Blood plasma and drawn blood volumes in the rat. *Proc. Soc. Exper. Biol. & Med.* **46**: 188, 1947.

- <sup>12</sup> MILLER L. L. AND ALLINO, E. L. Effect of dl methionine l-cystine and dl isoleucine on utilization of parenterally administered dog hemoglobin suggestion for designing the composition of an ideal protein digest J Exp Med 85 55 1947
- <sup>13</sup> ROBSCHT ROBBINS F S MILLER, L. L., ALLINO, E. L. AND WHIPPLE G H Plasma substitutes human and animal globin related to production of hemoglobin and plasma protein dog hemoglobin utilization improved by methionine but not by isoleucine J Exp Med 83 355 1946
- <sup>14</sup> —, —, —, AND — Hemoglobin and red cell production in experimental hemorrhage anemia N Y Acad Sci Preprinted Manuscripts Conferences on Some aspects of red cell production and destruction Apr 19-20 1946
- <sup>15</sup> MILLER, L. L. ROBSCHT ROBBINS F S, AND WHIPPLE G H Hemoglobin and plasma protein Their relation to internal body protein metabolism J Exp Med 81 405 1945
- <sup>16</sup> PLUMIER, M. Influence de l'injection intra-veineuse d'hémoglobine et de méthémoglobine sur le plasmatique chez le chien Compt rend Soc de Biol 140 589 1946
- <sup>17</sup> — Influence de l'injection intra-veineuse d'hémoglobine sur le fer biliaire chez le chien Compt rend Soc de Biol 140 805 1946
- <sup>18</sup> ROSS J F Hemoglobinemia and the hemoglobiurias New England J Med 233 691 1945
- <sup>19</sup> CONCORAN, A. C., AND PAGE I H Renal damage from ferrohem pigments myoglobin hemoglobin hematin Texas Rep Biol & Med 3 528 1945
- <sup>20</sup> LAMSON, P D ROBBINS B H AND GREIG M. E. Studies on shock induced by hemorrhage, hemoglobin solutions as blood substitutes J Pharmacol & Exp Therap 83 225 1945
- <sup>21</sup> WERTENBERGER G E AND HATKEBRING R Cardiovascular effects of a plasma substitute ('H.E.S. solution') in experimental hemorrhagic hypotension Fed Proc 4 76 1945
- <sup>22</sup> YUILL C L. GOLD M. A. AND HINDS E. G. Hemoglobin precipitation in renal tubules A study of its causes and effects J Exp Med 82 361 1945
- <sup>23</sup> BARNARD R D Pharmaceutical utilization of materials derived from human erythrocytes J Am. Pharm A. 34 177 1945
- <sup>24</sup> ALBANESE, A. A. Studies on human blood proteins I The isoleucine deficiency of hemoglobin J Biol Chem 157 613 1945
- <sup>25</sup> ORTEN J M., BOURQUE J E. AND ORTEN A U. Inability of human or beef globin to support normal hematopoiesis in rats without added isoleucine J Biol Chem 160 435 1945
- <sup>26</sup> DEVLIN, H B AND ZITTLE C A Nutritional study of human globin in rats J Biol Chem. 156 393 1944
- <sup>27</sup> BING R J Effect of hemoglobin and related pigments on renal functions of normal and acidotic dogs Bull Johns Hopkins Hosp 74 161 1944
- <sup>28</sup> RHODE C M., JENNINGS J E., AND AMBERSON W R. unpublished manuscript
- <sup>29</sup> CONANT, J B An electrochemical study of hemoglobin J Biol Chem 57 401 1913
- <sup>30</sup> AMBERSON W R JACOBS J E AND HISEY A. Human hemoglobin solutions as a blood substitute Fed Proc 1 3, 1942
- <sup>31</sup> JACOBS, J E, HISEY, A., AND AMBERSON, W R. Preservation of human hemoglobin solutions without methemoglobin formation Fed Proc 1 43, 1942
- <sup>32</sup> KEILIN D AND WANO Y L. Stability of hemoglobin and of certain endocytocytic enzymes in vitro Biochem J 41 491 1947
- <sup>33</sup> NEILL, J M. Studies on oxidation reduction of hemoglobin and methemoglobin IV Inhibition of spontaneous methemoglobin formation J Exp Med 41 561 1925
- <sup>34</sup> AMBERSON W R Blood substitutes Biol Rev Cambridge Phil Soc. 22 48, 1937
- <sup>35</sup> BROOKS M. M. Methylene blue and hemoglobin derivatives in asphyxial poisoning Am J Physiol 114 160, 1935
- <sup>36</sup> HAUSCHILD F Die Wirkung des Katalysins (Thionin) bei der Methämoglobinvergiftung Arch exp Path Pharmacol 124 458 1937
- <sup>37</sup> JUNO, F Studien über Methämoglobinbildung über die Rückbildung des Methämoglobins bei Gegenwart von Alkohol, Arch exp Path Pharmacol 124 464 1939
- <sup>38</sup> DITTRICH P Über methämoglobinbildende Gifte Arch exp Path Pharmacol 29 247 1891-1892
- <sup>39</sup> SAKURAI, K. Über die Rückbildung des Methämoglobins. I Versuche in vitro Arch exp Path Pharmacol 107 287, 1925

- <sup>10</sup> — Über die Rückbildung des Methämoglobins II Durchblutungsversuche Arch exp Path Phar makol 109 198 214 1925
- <sup>11</sup> DRABKIN D L Maintenance of active hemoglobin—a function of erythrocytes Fed Proc 5 132, 1946
- <sup>12</sup> GUTMAN H R JANDORY, B J, AND BODANSKY, O The role of pyridine nucleotides in the reduction of methemoglobin J Biol Chem 169 145, 1947
- <sup>13</sup> SPICER, S S, HANNA, C H AND CLARK, A M *In vitro* methemoglobin reduction in intact erythrocytes Fed Proc 6 294 1947
- <sup>14</sup> WARBURG O, KUROWITZ, F AND CHRISTIAN W Über die katalytische Wirkung von Methelnyl blau in lebenden Zellen Biochem Ztschr 227 245, 1930
- — — AND — — — Über die Wirkung von Phenylhydrazine und Phenylhydroxylamine auf den Stoffwechsel der roten Blutzellen (Methode zur Messung des Stoffwechsels roter Blutzellen) Biochem Ztschr 242 170, 1931
- <sup>15</sup> SHAPOT V S The mechanism of biological reactivation of methemoglobin in erythrocytes Biokhimiya 3 430 1938
- <sup>16</sup> KIESE, M. Die Reduktion des Hämoglobins Biochem Ztschr 316 264 1944
- Die Erhaltung des Blutfarbstoffs im wirksamen Zustande Klin Wchschr 24/25 81, 1946
- <sup>17</sup> WENDEL, W B Methylene blue, methemoglobin and cyanide poisoning J Pharmacol & Exp Therap 54 283, 1935
- <sup>18</sup> — Oxidation by erythrocytes and the catalytic influence of methylene blue I The oxidation of lactate in pyruvate II Methemoglobin and the effect of cyanide J Biol Chem 102 373 385, 1933
- <sup>19</sup> — Mechanism of antidotal action of methylene blue in cyanide poisoning Science 80 381, 1934
- <sup>20</sup> HUMMERICH, F Die Regulierung der Sauerstoff abgabe von Erythrocyten Biochem Ztschr 284 146, 1936
- <sup>21</sup> PENNELL, R B, SMITH W E, AND WERKHEISER, W C Preparation of hemoglobin solutions containing hemoglobin reducing enzymes Proc Soc Exper Biol & Med 65 295, 1947
- <sup>22</sup> EVERTY K. A, AND MALLOY, H T Determination of oxyhemoglobin methemoglobin and sulf hemoglobin in a single sample of blood J Biol Chem 126 655 1938
- <sup>23</sup> PHILLIPS R A personal communication 1944 HAMILTON, P B, personal communication, 1945, ANDERSON, W R., personal communication 1945
- <sup>24</sup> BARNES R B RICHARDSON, D, BERRY, J W AND HOOD, R L Flame photometry A rapid analytical procedure Ind Eng Chem Anal Ed 17 605 1945
- <sup>25</sup> BENEDICT S R Analysis of whole blood, determination of sugar and of saccharoids (non fermentable copper-reducing substances) J Biol Chem 92 141 1931
- <sup>26</sup> EMADEN G Eine gravimetrische Bestimmungsmethode für kleine phosphorsäuren Ztschr physiol Chem 113 138, 1921
- <sup>27</sup> FETTER, W J Gravimetric estimation of phosphates of blood phosphates in nephritis Arch Int Med 31 413 1923
- <sup>28</sup> WARBURG O Über Beeinflussung der Oxydationen im lebenden Zellen nach Versetzen an roten Blutkörperchen Ztschr physiol Chem 69 452 1910
- <sup>29</sup> RAPAPORT S Dimensional, osmotic and chemical changes of erythrocytes in stored blood I Blood preserved in sodium citrate neutral and acid citrate glucose (ACD) mixtures II Evaluation of several acid and neutral preservation mixtures Effect of storage at 25°C in Alsever's solution III Comparison of 3 dilutions of acid citrate glucose solution (ACD) IV Cells separated from plasma. J Clin Investigation 26 591 616 612, 629 1947
- <sup>30</sup> DICKEYS F The toxic effects of oxygen on brain metabolism and on tissue enzymes 1 Brain metabolism 2. Tissue enzymes Biochem J 40 145 171 1946
- <sup>31</sup> BARROV E. S G Studies on blood cell metabolism Effect of methylene blue on oxygen consumption of eggs of sea urchin and starfish mechanism of action of methylene blue on living cells J Biol Chem 81 445 1929
- <sup>32</sup> — — — AND HARROP G A JR Studies on blood cell metabolism effect of methylene blue and other dyes upon glycolysis and lactic acid formation of mammalian and avian erythrocytes J Biol Chem 165 1928



- <sup>66</sup> HIMMERICH F, AND FEINBERG R S Die Regulierung der Sauerstoffabgabe von Erythrocyten Blut glykolyse und Sauerstoffabgabe von Erythrocyten Biochem Ztschr 234, 152, 1936
- <sup>67</sup> WARBURO O AND CHRISTIAN W Über Aktivierung der Robisonischen Hexose Mono-Phosphorsäure in Roten Blutzellen und die Gewinnung aktivierender Fermentlösungen Biochem Ztschr 242, 206, 1931
- <sup>68</sup> IRVING J T Degradation of glucose by the blood corpuscle of the rabbit Biochem J 20, 613, 1926
- <sup>69</sup> WARBURO O Über Beeinflussung der Sauerstoffatmung Ztschr physiol Chemie 70, 413, 1911
- <sup>70</sup> GRAFE E. Über die Wirkung von Ammoniak und Ammoniakderivativen auf die Oxydationsprozesse in Zellen Ztschr physiol Chemie 79, 421, 1912.
- <sup>71</sup> MANN P J G AND QUASTEL, J H Nicotinamide cozymase and tissue metabolism Biochem. J 35, 502, 1941
- <sup>72</sup> HARROP G A JR AND BARRON E S G Studies on blood cell metabolism I The effect of methylene blue and other dyes upon the oxygen consumption of mammalian and avian erythrocytes J. Exp. Med 48, 207, 1928
- <sup>73</sup> WENDEL W B AND SHAFER P A Induced oxidation in blood Oxidation of lactic to pyruvic acid by methylene blue J Biol Chem 87, xx, 1930
- <sup>74</sup> WARBURO O, LUBOWITZ, F, AND CHRISTIAN W Kohlenhydratverbrennung durch Methämoglobin (Über den Mechanismus einer Methylenblaukatalyse) Biochem Ztschr 221, 494, 1930.
- <sup>75</sup> MICHAELIS L, AND SALOMON K. Methämoglobin Erzeugung und Atmungssteigerung durch organische Farbstoffe Biochem Ztschr 234, 107, 1931
- <sup>76</sup> BIRD R M Glycolysis in human blood J Biol Chem 169, 493, 1947

# THE EFFECT OF COBALT ON THE OXYGEN CAPACITY AND THE METHEMOGLOBIN CONTENT OF THE BLOOD

By MARY C. BUCCIERO, M.S., AND JAMES M. ORTEN, Ph.D.

ONE THEORY as to the mechanism of the production of polycythemia in the rat, and several other species, by cobalt, is that this substance interferes with cellular oxidative processes.<sup>1,4</sup> One way in which such an effect might be produced would be by an interference with the transport of oxygen in the blood to the cells either by a decrease in the oxygen capacity of hemoglobin itself or by the formation of methemoglobin possibly containing cobalt in place of iron. In the present investigation a study was made of the oxygen capacity and the methemoglobin and cobalt content of the blood of rats which had been maintained in a state of polycythemia by cobalt administration for a period of at least six weeks.

## EXPERIMENTAL

Male, weanling, albino rats Connecticut Agricultural Experimental Station strain, were used. They were fed an adequate synthetic basal diet described in a previous publication.<sup>2</sup> Various supplements were added to the basal diet in amounts also described in detail in the above paper. The groups studied included a control group and groups given cobalt alone (477 mg recrystallized  $\text{CoSO}_4 \cdot 7\text{H}_2\text{O}$  per kilo of diet), or cobalt supplemented with either choline (2.0 Gm choline chloride per kilo of diet), or cysteine (1.56 Gm L-cysteine hydrochloride per kilo of diet). The latter two groups of animals were part of a different study to be reported later. The amount of cobalt sulfate added to the diet supplies each rat with approximately 1.0 mg cobalt per day, an amount found in previous studies to produce a definite polycythemia in the rat. After the animals had been on experiment for a period of twenty weeks and the cobalt-treated rats had developed the characteristic polycythemia, with the exception of those given cysteine, as will be described in a subsequent publication, they were sacrificed and samples of blood were taken for analysis in the following manner. The animals were anesthetized with ether and five to eight ml of blood was drawn from the heart into tubes containing heparin. Of this amount, a very small portion was used for the total hemoglobin determination by the acid hematin method using the Coleman spectrophotometer. One ml was used for the determination of oxygen capacity, 1 ml for the estimation of methemoglobin, and the remainder was reserved for a spectrographic analysis\* for cobalt. The method used for the determination of the oxygen capacity of the blood was Sendrov's modification using the Van Slyke-Neill manometric appa-

From the Department of Physiological Chemistry Wayne University College of Medicine, Detroit.  
The data in this paper were taken from a dissertation presented by Mary C. Bucciero in partial fulfillment of the requirements for the degree of Master of Science, Wayne University, 1948.  
A preliminary report was made before the American Society of Biological Chemists at the Chicago meeting, May 1947.

Acknowledgment is expressed to Dr. Virginia Sink of the Research Laboratories, Chrysler Corporation, Detroit, for running the spectrographic analyses of the samples.

ratus.<sup>3</sup> The methemoglobin content of the samples was determined by a colorimetric procedure outlined in Kolmer and Boerner.<sup>3</sup> The remainder of the blood was carefully ashed and a spectrographic analysis for cobalt was made.

### RESULTS AND DISCUSSION

In Tables 1 and 2 are recorded the average terminal hemoglobin values, as determined by the acid-hematin and oxygen-capacity methods, and the average methemoglobin content of the blood of the various groups of animals. It is evident from the data in the two tables that there is a close correlation between the hemoglobin values by the two methods and that there is no significant amount of methemoglobin present in the blood of the cobalt-treated rats. The greater variations from

TABLE 1.—Terminal Hemoglobin Values of Control and of Cobalt-treated Rats

Group	Number of rats	Hemoglobin—Gm. %			
		Acid Hematin Method		O <sub>2</sub> Capacity Method	
		Average	Standard Deviation	Average	Standard Deviation
Control	8	15.4	±0.4	15.5	±0.5
Cobalt	8	19.7	±1.9	19.7	±2.1
Cobalt + Choline	7	19.2	±1.4	19.6	±1.3
Cobalt + Cysteine	8	17.4	±1.7	17.1	±1.5

TABLE 2.—Methemoglobin Content of Blood of Control and Cobalt-treated Rats

Group	Number of Rats	Total Hemoglobin Average Gm. %	Active Hemoglobin (by O <sub>2</sub> Capacity) Gm. %	Methemoglobin	
				Gm. %	Range
Control	8	15.3	15.6	-0.3	0.0 to -1.3
Cobalt	8	20.8	19.6	+1.2	+2.8 to -1.9
Cobalt + Choline	7	19.2	19.6	-0.4	+1.4 to -2.6
Cobalt + Cysteine	7	18.7	17.1	+1.6	+2.2 to 0.0

the average in the terminal hemoglobin and methemoglobin values observed in the cobalt-treated rats, as compared with the controls, appears to be a result of greater difficulties in obtaining and measuring blood samples in the former groups. The blood of the rats given cobalt was extremely viscous. The spectrographic analyses of the ashed blood samples showed no more than trace amounts of cobalt in any specimen.

The foregoing observations together thus constitute evidence that the mechanism of the production of polycythemia by cobalt is not one of the formation of an altered type of hemoglobin having a decreased oxygen-carrying capacity, nor can it be attributed to the formation of methemoglobin. Further substantiation of this view is afforded by the results obtained in the spectrographic analysis for cobalt which demonstrated the absence of more than a trace of that element in the blood. This latter observation is in agreement with that of Stare and Elvehjem<sup>6</sup> who found

only traces of cobalt in the blood of cobalt-treated polycythemic rats by a colorimetric method using nitroso-R-salt

### CONCLUSIONS

A study has been made of the oxygen capacity and the methemoglobin and cobalt content of the blood of rats administered approximately 1 mg cobalt daily for twenty weeks, in order to produce a sustained polycythemia.

No evidence of a decrease in the oxygen capacity of the blood of the cobalt-treated polycythemic rats was found, nor did the methemoglobin content differ significantly from the small amount found in the blood of control rats. No more than traces of cobalt were found in the blood of either group by spectrographic analysis.

These observations are interpreted as evidence that the mechanism of the production of polycythemia by cobalt is not one of lowering the oxygen capacity of hemoglobin nor of producing a methemoglobin, possibly containing cobalt rather than iron in the hemoglobin molecule.

### REFERENCES

- <sup>1</sup> BARRON A. G. AND BARRON E. S. G. Mechanism of cobalt polycythemia. Effect of ascorbic acid. *Proc Soc Exper Biol & Med* 35 407, 1936.
- <sup>2</sup> BUCCIERO M. C. AND ORTEN J. M. Choline and the production of polycythemia by cobalt in the rat. *Am J Physiol* 154 513, 1948.
- <sup>3</sup> KOLMER J. A., AND BOERNER F. *Approved Laboratory Technique*, ed. 4. New York: D. Appleton Century, 1945.
- <sup>4</sup> ORTEN J. M. On the mechanism of the hemopoietic action of cobalt. *Am J Physiol* 114 414, 1936.
- <sup>5</sup> PETERS J. P. AND VAN SLYKE, D. D. *Quantitative Clinical Chemistry*. Vol. II. Methods, ed. 1. Baltimore: Williams & Wilkins, 1932.
- STARR F. J. AND ELVEHJEM C. A. Cobalt in animal nutrition. *J Biol Chem* 99 473, 1933.

## EDITORIAL

### TRACE METALS IN BLOOD, WITH PARTICULAR REFERENCE TO ZINC AND CARBONIC ANHYDRASE

THE PHYSIOLOGIC role of the trace metals was neglected in biochemical investigations of the blood in the last decade, but recently interest has been aroused in their function in blood formation. The term trace metals is unfortunate in that it has carried the implications that these elements cannot be measured quantitatively, that they probably occur accidentally, and that their presence is of no discernible consequence.

The development of precise microchemical methods has given the whole study new impetus even though many of these methods are complex and difficult. The needs of industry have given rise to the formulation of excellent colorimetric, flame photometric, polarographic, and emission spectroscopic techniques, which are accurate for the measurement of very small quantities of elements, provided the necessary precautions regarding scrupulous cleanliness and avoidance of contamination of reagents and glassware are observed. Although the availability of radioactive isotopes has further stimulated interest in the field, it is erroneous to conclude that these substances serve their greatest usefulness in replacing microchemical techniques, actually, the two serve best as mutually interdependent but supplementary approaches.

The investigation of iron metabolism has been for years an important part of hematologic research. The association of iron with hemoglobin and porphyrin pigments lends itself readily to spectrophotometric analysis, thus making it a rewarding subject for study, especially during periods when good microchemical techniques for iron itself were not available. Knowledge concerning its metabolism is therefore developed to a degree unique among the metals. On the other hand, the relation of iron to the function of cytochromes, catalase and peroxidases in the erythrocyte is not established although it is known that these enzymes contain iron. Earlier physiologic work on cobalt and polycythemia<sup>1</sup> has been re-examined recently in the light of new data on anemias, particularly in relation to vitamin B<sub>12</sub>, a cobalt-containing complex. After previous work,<sup>2</sup> copper, too, is being studied in relation to regeneration of hemoglobin and in regard to its role in the hemocuprein found in red blood cells.<sup>3</sup> These elements are the only ones to have been studied with any degree of thoroughness. That titanium or vanadium are possible constituents of erythrocytes arouses no more than mild curiosity, although there is no reason for such an attitude other than the lack of information.

Available facts favor the assumption that many of the trace elements serve their function in association with protein molecules bound by S-H, COOH, NH<sub>2</sub>, or porphyrin groups. These proteins may be involved in hormone or enzyme systems (including vitamins) or may serve in functions of storage or transport. These metallo-enzyme systems are now receiving an increasingly large amount of attention.

An example of the rapid development of knowledge regarding a trace element is afforded by zinc, recent studies in the distribution and physiologic role of zinc are the result of advances in the chemistry of the rarer biologic elements and in nuclear physics and enzyme chemistry. It has been found that Zn is a constituent of human blood and is found in corpuscles and plasma. These studies were stimulated by the finding that Zn is more concentrated in leukocytes than in erythrocytes, as revealed by emission spectrography.<sup>5</sup> Subsequently, measurements have been made by means of a colorimetric technic<sup>6</sup> which is accurate to one microgram. The normal mean whole blood Zn level for males and females is 880 micrograms per cent, the normal mean plasma Zn 300 micrograms per cent, and 100 cc of packed erythrocytes contain 1440 micrograms.<sup>7</sup> Overall, 75 per cent of the whole blood zinc is found in the red cells, 22 per cent in the plasma and 3 per cent in the leukocytes separated from whole blood by a method based on physical chemical principles.<sup>8</sup> While a greater fraction of the total blood Zn is contained in the erythrocytes, cell for cell the leukocytes contain 25 times as much as the erythrocytes. Statistical analysis of the data suggest that zinc is a physiologic constituent of blood in that its individual variations in concentration follow the pattern of commonly observed biologic distribution phenomena. Actually, the metal occurs in quantities in the body which in modern biologic language can hardly be called traces.

Injection of radioactive  $^{65}\text{Zn}$  demonstrated its incorporation into the red and white blood cells of dog and man where it could be found as much as eight months after injection. Its passage across the placenta of the dog into the young has also been shown,<sup>9</sup> and might indicate its physiologic need.

The nature of the protein to which Zn is bound in plasma is unknown at present, although preliminary investigations<sup>10</sup> have shown that the metal becomes attached to the iron binding globulin<sup>11</sup> in vitro. However, it is not known whether this is the transport mechanism in the body.

The role of zinc in leukocytes is a mystery at present. Its differential distribution among the various groups of white cells has not been studied. Attempts at radioactive tagging in order to study the leukocytic life span have at best been inconclusive. The possible occurrence of exchange of zinc across the white cell membrane contributes to the difficulties of interpretation, and, most important of all, the nature of the compound with which Zn is associated in the leukocyte is unknown. The decrease of Zn in the peripheral leukocytes of patients with chronic myelocytic, lymphocytic and monocytic leukemia is, however, a startling abnormality. The concentration of zinc in the leukocytes of these patients is approximately 10 per cent of that found in normal leukocytes. Under therapy with x-ray or urethane and in clinical remission, the falling leukocyte count is accompanied by a rise of zinc to normal levels. Attempts at raising the Zn level of these cells and lowering the leukocyte count by injections of stable zinc gluconate have not been successful.<sup>1</sup> Whether leukemic cells are Zn deficient because they are immature, or whether they are leukemic because they are Zn deficient is difficult to evaluate at present. However, investigations of mouse epidermis<sup>12</sup> have shown a decrease in

Zn and other elements as neoplasia developed. This is at least a clue to the fact that there is a rearrangement of the rarer elements in neoplastic tissues. It is evident that it will be necessary to study all of the minor elements in leukemic cells before any conclusions concerning the true meaning of the decreased Zn content may be drawn. Unquestionably, however, studies of Zn metabolism offer a new approach to leukopoiesis.

More is known concerning zinc in erythrocytes. Keilin and Mann<sup>14</sup> recorded the presence of that element in the enzyme carbonic anhydrase, shown earlier by Meldrum and Roughton<sup>15</sup> and by Stadie and O'Brien<sup>16</sup> to be contained in the red blood cell. The enzyme has a molecular weight of approximately 30,000 and contains 0.3 per cent zinc. The functional significance of the zinc in the molecule is shown by the fact that removal or inactivation of the metal by trichloroacetic acid or by BAL<sup>17</sup> also inactivates the enzyme. The enzyme catalyzes the reaction  $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3$  which otherwise would proceed at too slow a rate to permit life in mammals or birds. It is evident that carbonic anhydrase may be as important in carbon dioxide transport as hemoglobin is in oxygen transport, yet the former has not been investigated thoroughly. Studies of the enzyme in human and dog blood have shown that all the activity is in the erythrocytes. There is none in plasma or leukocytes. *It is evident, therefore that zinc exists in blood in several states.*

The carbonic anhydrase content and Zn concentration of the red cells parallel each other and vary directly with the hematocrit level and the hemoglobin concentration in congestive failure, anemia and polycythemia.<sup>18-22</sup> It is of unusual interest, however, that in pernicious anemia the erythrocyte zinc concentration and blood carbonic anhydrase activity are in or close to the normal range despite low hematocrit, cell percentages and hemoglobin values, the mean corpuscular zinc concentration and carbonic anhydrase activity are increased several times more than the high mean corpuscular hemoglobin and out of all proportion to the increase in cell size. Normal findings develop in remission in a period of time commensurate with the known mean life span of pernicious anemia red cells, evidently as a result of their being replaced by normal cells.<sup>19-22</sup> In other clinical conditions such as the postnatal state in infants,<sup>23</sup> the sickling phenomenon<sup>21</sup> and paroxysmal cold hemoglobinuria<sup>25</sup> studies of the carbonic anhydrase system suggest a role of the enzyme in their mechanisms.

Although the above findings relating to disturbances in enzyme activity and zinc concentration in various conditions are of interest, their significance cannot be stated. At present, sufficient data are not available on the physiology of carbonic anhydrase to make possible a definition of its precise function in the blood. Thus, although it is known what the enzyme can do, what it actually does is not known. However, the observation that a close parallelism exists between the erythrocyte carbonic anhydrase activity and zinc content in all conditions studied<sup>19</sup> provides a useful method for estimating the amount of the enzyme in the red blood cells, apparently all the zinc in erythrocytes is part of the carbonic anhydrase molecule.

The present data bearing on zinc in the blood, although incomplete, call atten-

tion to the even larger gaps which exist in knowledge of other erythrocyte metals and metallo-enzyme systems

BERT L. VALLEE, M.D.\*

MARK D. ALTSCHULE, M.D.

Boston, Massachusetts

# REFERENCES

- <sup>1</sup> ORTEN, J. M., UNDERHILL, F. A., MUORAGE E. R., AND LEWIS, R. C. Polycythemia in the rat on a milk iron-copper diet supplemented by cobalt. *J Biol Chem* 96 11, 1932.
- <sup>2</sup> RICKES, E. L. BRINK, N. G., KONTUSZY F. R., WOOD, T. R., AND FOLKERS, KARL. Vitamin B<sub>12</sub>: a cobalt complex. *Science* 168 134 1948
- <sup>3</sup> ELVEHJEM, C. A. The biological significance of copper and its relation to iron metabolism. *Physiol Rev* 15 471 1935
- <sup>4</sup> KEILIN, D., AND MANN, T. Haemocuprein and hepatocuprein, copper protein compounds of blood and liver in mammals. *Proc Roy Soc B* 126 303, 1938
- <sup>5</sup> VALLEE, B. L., AND FLUHARTY, R. G. Zinc metabolism studied by means of the radioactive isotope <sup>65</sup>Zn. *J Clin Investigation* 26 1199 1947
- <sup>6</sup> — AND GIBSON, JOHN G. 2ND. An improved dithionite technique for the determination of small quantities of zinc in blood and tissue samples. *J Biol Chem* 176 435 1948
- <sup>7</sup> — AND —. The zinc content of normal human whole blood, plasma, leucocytes and erythrocytes. *J Biol Chem* 176 445 1948
- <sup>8</sup> — HUGHES, W. L., AND GIBSON, JOHN G. 2ND. A method for the separation of leucocytes from whole blood by flotation on serum albumin. *Blood Special Issue No. 1* 82, 1947
- <sup>9</sup> —, FLUHARTY, R. G. AND GIBSON, JOHN G. 2ND. Distribution of zinc in normal blood and organs studied by means of <sup>65</sup>Zn. Program of the IVth International Cancer Research Congress. St. Louis 1947. Page 137 (Reprinted in the Proceedings of the Congress)
- <sup>10</sup> —, AND KOECHLIN, B. A. Unpublished data
- <sup>11</sup> SURGENOR, D. M., KOECHLIN, B. A., AND STROMO, L. E. Chemical, clinical and immunological studies of the products of human plasma fractionation XXXVII. The metal combining globulin of human plasma. In Press, *J Clin Investigation*
- <sup>12</sup> GIBSON, J. G. 2ND, VALLEE, B. L., FLUHARTY, R. G., AND NELSON, J. E. Studies of the zinc content of the leucocytes in myelogenous leukemia. Program of the IVth International Cancer Research Congress. St. Louis, 1947. Page 104. (Reprinted in the Proceedings of the Congress)
- <sup>13</sup> CARRUTHERS, C. AND SUNTZEFF, V. Copper and Zinc in epidermal carcinogenesis induced by methylcholanthrene. *J Biol Chem* 159 647 1945
- <sup>14</sup> KEILIN, D. AND MANN, T. Carbonic anhydrase. Purification and nature of the enzyme. *Biochem J* 34 1163, 1940
- <sup>15</sup> MELDRUM, N. U. AND ROUGHTON, F. J. W. Carbonic anhydrase. Its preparation and properties. *J Physiol* 86 113 1933
- <sup>16</sup> STADIE, W. C. AND O'BRIEN, H. The catalysis of the hydration of carbon dioxide and dehydration of carbonic acid by an enzyme isolated from red blood cells. *J Biol Chem* 103 521, 1933
- <sup>17</sup> WEBB, E. C. AND VAN HEYMOEN, R. Action of British anti-lewisite (BAL) on enzyme systems. *Biochem J* 41 74 1947
- <sup>18</sup> ALTSCHULE, M. D., AND LEWIS, H. D. Blood carbonic anhydrase activity in patients with dyspnea consequent to chronic cardiac and pulmonary disease. *Arch Int Med* In press
- <sup>19</sup> VALLEE, B. L., LEWIS, H. D., ALTSCHULE, M. D. AND GIBSON, JOHN G. 2ND. The relation between carbonic anhydrase activity and zinc concentration of erythrocytes in normal and in anemic and other pathological conditions. *Blood* in press

\* Senior Research Fellow. National Research Council. Committee on Growth supported by the American Cancer Society



- <sup>9</sup> LEWIS H D AND ALTSCHULE M D Blood carbonic anhydrase activity in anemia With a note on polycythemia vera Blood in press
- <sup>10</sup> VALLEE B L AND GIBSON JOHN G AND The zinc content of whole blood plasma leucocytes and erythrocytes in the anemias Blood in press
- <sup>11</sup> ——— Zinc and carbonic anhydrase content of red cells in normals and in pernicious anemia J Clin Investigation 27 559 1948
- <sup>12</sup> ALTSCHULE M D AND SMITH C A Carbonic anhydrase activity of the blood of newborn infants To be published
- <sup>13</sup> TOMLINSON W J AND JACOB J E Studies of sickle-cell formation in normal saline plasma and sera with carbonic anhydrase inhibitors J Lab & Clin Med 31 107 1945
- <sup>14</sup> WAOLEY P F ZINKRAM W H AND SIEBENS A A A note on studies of hemolysis in paroxysmal (cold) hemoglobinuria Am J Med 2 342 1947

## NEWS AND VIEWS

### BLOOD CLUB

The second annual meeting of the Blood Club will be held in Atlantic City on May 1, 1949 on the Sunday evening just prior to the meetings of the American Society of Clinical Investigation and the Association of American Physicians Dinner will be served promptly at 6 30 P M in Haddon Hall Hotel after which the following preliminary program, in the form of a panel discussion on Hemorrhagic Disorders Associated with Defective Coagulation, has been arranged

#### A Introduction

#### B Discussion of the Abnormal Mechanisms in Coagulation in the Following Conditions

##### 1 Hypoprotirembinemia

- |                                                                   |                              |
|-------------------------------------------------------------------|------------------------------|
| a Idiopathic and/or Congenital                                    | c Secondary to liver disease |
| b Secondary to Intestinal Absorption and/or to Dietary Deficiency | d Secondary to Dicumarol     |

Discussors h M Brinkhous W H Seegers L M Tocantins

##### 2 Deficiencies in Platelet Material

- |                |                  |
|----------------|------------------|
| a Thrombopenia | b Thrombasthenia |
|----------------|------------------|

Discussors h M Brinkhous W H Seegers L M Tocantins

##### 3 Hemophilia

Discussors K M Brinkhous C G Craddock F L Munro L M Tocantins

##### 4 Deficiency in Accelerator Globulin

Discussors K M Brinkhous W H Seegers

##### 5 Circulating Anti-Coagulants

- |                    |                          |
|--------------------|--------------------------|
| a Hemophilia       | c Anaphylactic Shock     |
| b Idiopathic       | f Secondary to H-parin   |
| c Radiation        | g Secondary to Dicumarol |
| d Nitrogen Mustard |                          |

Discussors J Garrott Alleo C L Conley C G Craddock Leon Jacobson F L Munro

This meeting is open to all interested physicians Reservations for the dinner must be made by writing to Dr Lawrence The dinner charge will be at regular hotel prices

The Committee John S Lawrence, University of California, at Los Angeles  
Frank Bethell, Ann Arbor  
Joseph F Ross, Boston

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## *Contributors to this Issue*

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### MARK D. ALTSCHULE, M.D.

Assistant Professor of Medicine, Harvard Medical School; Director of Internal Medicine and Research in Clinical Physiology, McLean Hospital, Waverly, Mass.; Visiting Physician and Associate in Research, Beth Israel Hospital, Boston, Mass.

### D. ANDREUCCI, M.D.

Department of Obstetrics and Department of Histology and Embryology, Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brazil.

### ARNOLD R. AXELROD, M.D.

Teaching Fellow in Medicine, Wayne University College of Medicine, Detroit, Mich.

### COMMANDER W. W. AYRES, MC, U.S. NAVY

Chief Pathologist, Naval Medical School, Medical Center, Bethesda, Md.

# CONTRIBUTORS TO THIS ISSUE

## LAWRENCE BERMAN, M D

Associate Professor of Pathology and Associate in Medicine, Wayne University College of Medicine Detroit, Mich Consultant, Anemia Laboratory Out Patient Department Harper Hospital Consultant Anemia Laboratory Wayne County General Hospital Hematology Consultant, Veterans Hospital, Dearborn, Mich

## A BLAUSTEIN, M D

Lecturer in Pathology, University of Vermont Burlington Vt, Formerly of the Department of Hematology Royal Victoria Hospital Montreal, Quebec, Canada

## LEE BURNETT, M S

Laboratory for Experimental Cytology Lenox Hill Hospital New York, N Y

## W B CASTLE, M D

Professor of Medicine Harvard University Director, Thorndike Memorial Laboratory and Director, Second and Fourth Medical Services (Harvard), Boston City Hospital Boston Mass

## TALBERT COOPER, M D

Instructor in Medicine Mayo Foundation, Graduate School of the University of Minnesota Rochester Minn

## WILLIAM DAMESHEK, M D

Hematologist J H Pratt Diagnostic Hospital Professor of Clinical Medicine Tufts College Medical School Boston, Mass

## EDWARD W DEMPSEY, PH D

Associate Professor of Anatomy, Harvard Medical School Boston, Mass

## THEODORE S EVANS, M D

Attending Physician Hospital of St Raphael New Haven Conn

## ROBERT S FADEM, M D

Resident in Medicine Mercy Hospital San Diego Calif

## ELEANOR M FLEMING, A B

Laboratory Technician Thorndike Memorial Laboratory Boston City Hospital Boston Mass

## VICTORINO DE LA FUENTE, M D

Assistant Professor in Clinical Medicine and Chief of Hematology Service University of Santo Tomas Clinical Pathologist Clinica Singian Consulting Hematologist Clinica Lopez, Manila Philippine Islands

## J L GABRILOVE, M D

Blumenthal Fellow in Medicine, Mount Sinai Hospital New York, N Y

## ROBERT P GIBB, M D

Assistant in Medicine Yale University School of Medicine, New Haven, Conn

## JOHN G GIBSON, II, M D

Research Associate in Medicine Harvard Medical School Associate in Medicine Peter Bent Brigham Hospital, Boston, Mass

## S GRANICK, PH D

Laboratories of the Rockefeller Institute for Medical Research New York N Y

## RUTH E HODGSON, M S

Laboratory Technologist and Teaching Assistant (Hematology) Department of Anatomy University of Minnesota Minneapolis, Minn

## THOMAS N HORAN, M D

Assistant Professor of Clinical Medicine, Wayne University College of Medicine Detroit, Mich

## M JACOBS, A B

Technical Assistant Mount Sinai Hospital, New York, N Y

## SAMUEL D JACOBSON, M D

Assistant Professor of Medicine Wayne University College of Medicine Detroit, Mich

## NANNIE K M DELEEUW, M D

Archibald Fellow Department of Experimental Surgery McGill University Montreal Quebec, Canada

## HERBERT D LEWIS, M D

Assistant in Medicine Harvard Medical School Associate in Medical Research Beth Israel Hospital Boston Mass

## JONAH G LI, M D

Staff Hematologist Division of Experimental Medicine University of Oregon Medical School, Portland Ore

## ALLEN H MINOR, M D

Laboratory for Experimental Cytology Lenox Hill Hospital New York N Y, Postgraduate Research Fellow National Cancer Institute

## ROBERT R NESBIT, M D

Pathologist Hospital of St Raphael New Haven Conn

CONTRIBUTORS TO THIS ISSUE

EDWIN E OSGOOD, M A , M D

Professor of Medicine and Head Division of  
Experimental Medicine, University of Oregon  
Medical School, Portland Ore

R DOROTHY SUNDBERG, Ph D

Assistant Professor of Anatomy University of  
Minnesota Hematologist University of Minne-  
sota Hospital Laboratories Minneapolis Minn

M RABINOVITCH

Department of Histology and Embryology  
Faculdade de Medicina da Universidade de São  
Paulo São Paulo Brazil

BERT L VALLEE, M D

Research Fellow in Medicine Harvard Medical  
School Boston Now Research Associate, De-  
partment of Biology Massachusetts Institute of  
Technology Cambridge Mass

JACK J RHEINGOLD, M D

Formerly Fellow in Hematology J H Pratt  
Diagnostic Hospital Boston Mass present ad-  
dress Washington D C

M VOLTERRA, M D

Adjunct Physician Mount Sinai Hospital New  
York N Y Moritz Rosenthal Fellow in Medi-  
cine

ELWOOD A SHARP, M D

Director Department of Clinical Investigation  
Parke Davis & Co Director Anemia Labora-  
tory Out Patient Department Harper Hospital  
Associate in Medicine Wayne University College  
of Medicine Detroit Mich

ELMORE C VONDERHEIDE, M D

Anemia Laboratory Out Patient Department,  
Harper Hospital Instructor in Clinical Medicine,  
Wayne University College of Medicine Detroit  
Mich

SHU CHU SHEN, M D

Formerly Research Fellow Harvard Medical  
School Research Fellow, Thorndike Memorial  
Laboratory Assistant in Medicine Boston City  
Hospital Boston Mass Now Instructor in  
Medicine Tufts Medical School Assistant Visi-  
ting Physician Holy Ghost Hospital Cambridge  
Consultant Jewish Memorial Hospital and Junior  
Visiting Physician, Boston Dispensary Boston  
Mass

CHARLES H WATKINS, M D

Associate Professor of Medicine Mayo Founda-  
tion Graduate School of the University of Minne-  
sota Rochester, Minn

L J SOFFER, M D

Associate Physician Mount Sinai Hospital  
Assistant Clinical Professor of Medicine Columbia  
University New York N Y

STEWART H WEBSTER, Ph D

Senior Biochemist Laboratory of Physical  
Biology National Institutes of Health Bethesda  
Md

BERNHARD STEINBERG, M D

Director of Institute of Medical Research Patholo-  
gist to The Toledo Hospital Toledo Ohio

GEORGE B WISLOCKI, M D

Hershey Professor of Anatomy Harvard Medical  
School Boston Mass

ROBERT E STOWELL, M D

Professor of Pathology and Oncology University  
of Kansas Medical School Kansas City Kansas

HOWARD D ZUCKER, M D

Laboratories Division of Pathology Mount Sinai  
Hospital New York N Y Theodor Aschench  
Fellow in Pathology 1947

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# BLOOD

## *The Journal of Hematology*

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VOL IV, NO 5

MAY, 1949

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### PREFACE

#### BLOOD CELLS AND THEIR REACTIONS

TWO YEARS AGO, a special issue of *Blood*, Morphologic Hematology, was published. At that time *Blood* was a bimonthly publication, and purchase of this special issue placed an extra burden upon the interested subscriber. This was criticized sharply, and as a result it was determined that in the future, all special issues should be sent to regular subscribers without extra cost. The present issue represents the first application of this principle. The excess in cost above the publication charge for a regular issue has been borne by a very generous grant from the Lederle Laboratories Division of the American Cyanamid Company.

Despite the startling advances made in the physiologic and chemical aspects of hematology, the base line of that specialty must still remain the cell. The reactions of the blood cells, their enzymatic constituents, their chemical reactions are now being actively studied by such techniques as those of histochemistry and phase microscopy. In fact, the cytologist, far from having lost ground, is now in an enviable position. From the appearance and reactions of a cell when subjected to various chemical reactions, it is possible to interpret in some measure its complicated functions, and from this it is only a short step to phenomena of abnormal growth such as leukemia and leukosarcoma. The ultimate control of such highly proliferative diseases will come only through a knowledge of the complicated growth problems of the white cells.

In the past several months, a large number of manuscripts devoted to blood cells has been accepted for publication in *Blood*. In this issue, many of them are arranged together in broad sections dealing with the red cells, the leukocytes, and the platelets. It is hoped that this symposium of blood cells and their reactions will prove of value and will serve as a convenient reference to some of the modern phases of morphologic hematology.

WILLIAM DAMESHEK  
for the Editors of *Blood*

# THE CHEMISTRY AND FUNCTIONING OF THE MAMMALIAN ERYTHROCYTE

By S GRANICK, PH D

## INTRODUCTION

THE ERYTHROCYTE has been one of the most intensively studied of cells. Much of the data, however, remains isolated in specialized fields. It was with the intention of bringing together and correlating some aspects of these specialized fields that this review was undertaken. If it can serve to indicate the complexity and beauty of design of this bit of protoplasm as a unit, as well as in its constituent parts, it will have been worth-while.

## I DEVELOPMENT OF THE RED CELL

*Technic of Cytochemistry* The recent advances in the study of red cell development are due mainly to the newer technic of cytochemistry. The cytologist has for a long time made skillful use of various dyestuffs to study the changes in staining of particular regions of cells and to observe the changing staining capacities in the differentiation of the cells. Within recent years, interest has centered on the interpretation of staining in terms of the affinity of dyestuffs for special chemical groupings of substances. As might be expected, the major forces in these staining procedures are of coulombic nature. Thus, for example, basic dyestuffs, like methylene blue or brilliant cresyl blue, carrying positive charges, appear to stain acidic substances, carrying negative charges such as the phosphoric acid groups in nucleic acid or the half-sulfuric acid ester groups of heparin.<sup>1</sup> Again, acidic dyes like eosin, with negatively charged groups, tend to combine more firmly with the positively charged basic groups like those on histones or on globin. Supporting techniques have now placed these interpretations of staining on a firmer foundation and also have extended our chemical knowledge of the cell substances. Among such supporting techniques has been the Feulgen staining for desoxyribose which has shown that desoxyribose nucleic acid is limited to the chromatin of the nucleus. The intense absorption of the purine and pyrimidine components of nucleic acid in the region of 2,600 Å has been utilized by Caspersson in an ingenious technic to determine the location of these substances in specific regions of a cell. The use of enzymes for digesting away specific substances has shown the localization of materials such as proteins, ribose nucleic or desoxyribose nucleic acid, etc., in fixed cells. Advantage has also been taken of the enzymic action of the cells themselves in order to determine the location and activity of regions containing the particular enzyme.<sup>2-3</sup> In addition, the dye-binding capacity over a range of pH may permit of the identification of proteins of high or low isoelectric points.<sup>4</sup> Detailed studies of the red cell using some of the absorption spectra techniques of Caspersson<sup>5</sup> have recently been reported in a monograph by Thorell.<sup>6</sup>

From the Laboratories of The Rockefeller Institute for Medical Research, New York, N. Y.

*Cytochemistry of Red Cell Development* The transformation of a reticular cell of the bone marrow—a rather nondescript kind of a cell, relatively small, with a nucleus poor in chromatin—into the mature red cell, is brought about by the little understood processes of nucleic acid metabolism, of protein synthesis and of differentiation. Such processes are not peculiar to the red cell, but the manifestation of these processes is perhaps more readily apparent in this cell than in other cell types. Although it will be possible to say little with certainty about these processes, it will be useful to examine the development of the red cell to see what inferences may be drawn about these processes in chemical terms.

In the following description of the changes in red cell development the major data have been derived from the studies of Sabin<sup>7</sup> and of Thorell. According to Sabin, reticular cells of the bone marrow endothelium, which develop in sinusoids temporarily closed to circulation, give rise to the erythrocytic series. On the other hand, those reticular cells which develop extravascularly give rise to the granulocyte series. If this interpretation of origin is a correct one, then we have here a most interesting example of differentiation.\* From a chemical point of view the mechanism of this differentiation might be studied most readily in tissue culture by examining the effect of nutrition and environment on the kind of cell type which would arise. A method for the culture of marrow has been developed by Osgood and Brownlee<sup>8</sup> and recently was modified by Plum.<sup>9</sup>

Once the presumptive proerythroblast has been established, three phases of growth to the mature erythrocyte may be distinguished (fig. 1). The first is a phase of rapid cell multiplication, then occurs a decline in growth rate. Finally, overlapping with this decline, a marked differentiation sets in, hemoglobin being synthesized concomitantly with a loss of other cytoplasmic proteins and desoxy-ribose nucleic acids.

The stem cell of the erythrocytic series, the proerythroblast, is a cell 12  $\mu$  in diameter, containing a moderate number of mitochondria, a golgi net, a cytocentrum and conspicuous nucleoli. The chromatin of the nucleus contains desoxy-ribosenucleic acid. The nucleolus and the cytoplasm contain only ribosenucleic acid. The cytoplasm at this stage is high in ribose nucleic acid containing about 5 per cent of its dry weight in this substance (fig. 1-A). The high content of ribose-nucleic acid runs parallel with the basophilic staining which is maximum in the proerythroblast. At this stage, the cells multiply rapidly and one may therefore infer that there is taking place intense protein and nucleic acid synthesis both in the nucleus and in the cytoplasm.

In the second stage, that is, in the early or basophilic erythroblast, the concentration of cellular protein is at a maximum (fig. 1-B) and this is also reflected in the curve for the product of the number of cells times the cell volume, which attains a maximum (fig. 1-C). The basophilic erythroblast has a diameter now of 10  $\mu$  (fig. 1-E) the ribose nucleic content of the cytoplasm has decreased to 2 per cent, the nucleus has lost its nucleoli and contains angular particles of chromatin.

In the late or polychromatic erythroblast the ribose nucleic acid has further

\* For details of differentiation in the evolution of the blood forming tissues the account by Jordan<sup>126</sup> is recommended.



diminished, paralleling the decreased affinity of the cytoplasm for basic dyes (fig 1-A) At the same time, the staining with acid dyes becomes significant, which has been considered as indicating the increase in the relatively basic protein globin (fig 1-B)

In general, the cytologist has assumed that this increased intensity of staining with acid dyes can parallel with the development of the hemoglobin color of the cell, this would mean that globin and heme synthesis occurred simultaneously. Measurements by Thorell, using the strong absorption band of heme at 4,000 Å,

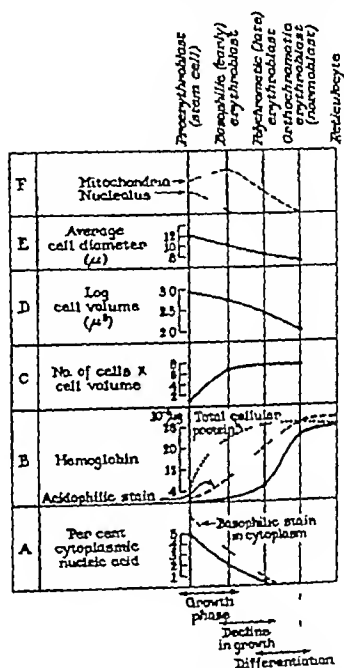


FIG. 1—THE DEVELOPMENT, DIFFERENTIATION AND MATURATION OF THE ERYTHROCYTE (modified from Sabin and from Thorell)

Quantitative data are presented by the full lines; qualitative changes by the broken lines.

have clearly demonstrated the rapid development of heme and presumably hemoglobin during the polychromatophilic erythroblast stage, no further increase occurring in the normoblast stage. Thorell considers that the rapid synthesis of cellular proteins is intimately connected with a high ribose nucleic acid content, as exemplified in the first stage of development (fig 1-A). According to Thorell the high cellular protein which is found at the end of the first stage might be globin itself, heme would be added to form hemoglobin only after the late erythroblast stage. However, if in the early erythroblast stage, the globin content were high, the cell ought to be stainable with acid dyes, which it is not (unless perhaps the high nucleic acid in the cytoplasm could form some kind of a complex with the

globin to prevent acidic staining.) If it is assumed that the staining technic reveals the presence of globin, then it would appear that at a relatively late stage, in a cytoplasm rich in proteins, these proteins would be gradually replaced almost completely and exclusively by a single kind of protein, globin, that is, the curve of hemoglobin synthesis would also represent the curve of globin synthesis (fig 1-B)

In the normoblast or orthochromatic erythroblast of about  $7\mu$  diameter, cell divisions no longer occur. The nucleus has become small and pyknotic, mitochondria have disappeared, and only a trace of ribose nucleic acid remains in the cytoplasm. However, the hemoglobin concentration has increased from 1-2 per cent of the previous stage up to 20 per cent at the normoblast stage where it remains constant (fig 1-B), the cytoplasm now stains intensely with acid dyes as would be expected from its high globin content. This stage of the normoblast lasts about two days.<sup>10</sup> Some time after the loss of the nucleus, there occurs the flattening and appearance of biconcavity of the cell. Plum has recently claimed that the normoblasts give rise to mature erythrocytes by a process of budding off droplets of cytoplasm.<sup>11</sup>

In the reticulocytes the stroma of the cell is 2.5-4.5 times greater than in the normoblast. Such reticulocytes contain more lipid than do the mature red cells and their surface is stickier.<sup>10</sup> The reticulocyte is of slightly larger volume and lesser density than the mature red cell. Dustin<sup>12</sup> has demonstrated, using ribonuclease, that the reticulum contains ribosenucleic acid. The change from the reticulocyte to the mature red cell occurs in from one to three days. According to the investigations of Plum,<sup>11</sup> there appears to be a thermolabile 'unripe' substance, especially high in concentration in the mucosa of the fundus stomach, which when carried to the reticuloendothelial system is activated by tyrosine, resulting in the formation of a fully ripe maturing substance present in blood plasma. This maturing substance, acting on reticulocytes, is claimed to cause the disappearance of the reticulum.

Thorell has found that nucleated megaloblast cells in pernicious anemia have a high content (4-5 per cent) of ribosenucleic acid in the cytoplasm and also contain hemoglobin, the hemoglobin content increasing as the size of the cell decreases. This may be contrasted with the polychromatophile erythroblast where very little ribose nucleic acid is present in the cytoplasm and the hemoglobin is increasing rapidly.

Since the metabolic end product of the nucleic acid purines is uric acid, the rapid maturation of erythrocytes should be accompanied by an increase in excretion of uric acid. This is most readily observed in recovery from pernicious anemia where approximately 10 Gm uric acid are excreted in the urine for an increase in count of 1 million erythrocytes per cu mm. The increase in uric acid in the blood starts within twenty-four hours after the onset of treatment and the peak of the uric acid precedes the peak of reticulocyte production by twenty-four hours.<sup>13</sup>

As the red cell matures it differentiates into what is essentially a tiny bag,  $7\mu$  in diameter, containing hemoglobin. During the differentiation the cell loses its nucleus, and with it the ability to make desoxyribosenucleic acid. In the reticulocyte stage the last vestiges of ribosenucleic acid are seen as the reticulum, and at this time when the ribosenucleic acid disappears there has disappeared the ability to

synthesize hemoglobin and heme.<sup>14</sup> In the mature red cell very little oxygen is utilized for respiration (0.05–0.1 cc oxygen per mg dry weight per hour as compared with ten times this rate for the nucleated erythrocyte<sup>15</sup>). From this fact it may be inferred that some portions of the cytochrome oxidase system are absent or nearly so. According to recent findings,<sup>16</sup> the cytochrome oxidase enzymes are generally contained in mitochondria. The fact that mitochondria have disappeared from the mature red cell, therefore, correlates well with its low oxygen metabolism. Small but important concentrations of certain other proteins are present. For example, an enzyme system remaining in the mature erythrocyte is the glycolytic system which appears to be a rather complete one. This system serves to maintain the hemoglobin in the reduced or functional ferrous state. In addition, catalase is present to protect the heme from peroxide decomposition and carbonic anhydrase is present to aid in the transport of  $\text{CO}_2$  as bicarbonate ions.

## II COMPOSITION OF THE WATER INSOLUBLE CONSTITUENTS, I E., THE STROMA

When red cells are hemolyzed, the soluble constituents flow away and there remains an insoluble residue of ghosts, the membranes of which have a thickness of some 200–300 Å. This material constitutes about 2–5 per cent of the wet weight of the original cell. Of the stroma material, some 40–60 per cent is insoluble protein and some 10–12 per cent is lipid.<sup>17, 18</sup> A method for the preparation of the stroma has been described by Parpart.<sup>19</sup>

*Properties of the Red Cell Surface.* Various properties, such as hemolysis with many different substances, selective permeability to anions, blood group materials, virus affinities, etc. indicate that the stroma is a complex or mosaic structure derived not only from the red cell proper, but also to some extent accumulated by accretion. The red cell is a reagent of great sensitivity for the detection of various substances if these substances can bring about agglutination or hemolysis. This sensitivity results from the fact that only relatively few discrete areas or specific groupings on the relatively large surface of the red cell need to be acted upon in order to observe hemolysis or agglutination. However, it has not been possible as yet to infer from the action of many of these reagents what specific groupings of the red cell membrane may be involved.

A subject of great interest at present is the mechanism by which some animal viruses bring about the agglutination of some species of red cells. For example, Hirst<sup>20</sup> first found that the influenza virus brings about the agglutination of human and chick red cells, and Horsfall et al.<sup>21</sup> have described the agglutination of mouse or hamster red cells by the pneumonia virus of mice. On chick red cells the receptor areas for influenza virus were found to be very stable to treatment with relatively high temperatures and with a number of reagents, but they were inactivated by proteolytic enzymes, by periodate, as well as by several hours contact with the influenza virus itself, (i.e., the virus appeared to possess enzymic activity). It was concluded that protein seemed to be an essential constituent of this receptor. According to Hirst, in the IV-4 fraction of normal serum, a  $\beta$  globulin is present, having certain properties which are similar to those of the receptor spot and this substance does not have the properties of a mucin. De Burgh et al.<sup>22</sup> have prepared,

from the erythrocytes, extracts which contain at least 50 per cent of polysaccharide and which are capable of inhibiting specifically the hemagglutinating action of the virus. The effect is presumably due to competitive combination of this extract with the virus. The virus appears to act on this extract, possibly enzymatically, as it does on the red cell surface, i.e., on prolonged contact of virus and the extract a change occurs in the extract so that it no longer binds the virus. The influenza inhibitor has also been obtained from human lung. If the virus is heated, agglutination of the red cells still takes place but the enzymic activity seems to be destroyed. It may be postulated either that the chemical groupings necessary for agglutination are destroyed by the enzyme or that the enzyme activity brings about new groupings which interfere with the groups taking part in the agglutination. Hanig<sup>22</sup> has found that the electrokinetic potential of a human red cell is maximally depressed when about 300 influenza virus particles coat the cell, covering only about one-eightieth of the surface. Ordinarily, the electric mobility of the red cell remains unchanged in the presence of various proteins, including even the anti-sphering protein of Furchgott.

Hemolysis, the escape of hemoglobin from the red cell under isotonic conditions, may be brought about by changes in different regions of the cell surface.<sup>15</sup> For example, surface active agents such as digitonin, saponin, lysolecithin, etc., may dissolve out fatty materials or may denature proteins in the cell surface, leaving holes sufficiently large for the hemoglobin molecules to diffuse out. Such agents may be effective in concentrations lower than would be needed to form a single monolayer on the surface. Still more effective agents are the specific immune bodies such as the agglutinins and hemolysins.

The hemolysins may react with the red cell to cause hemolysis directly. More commonly, hemolysis appears to be brought about indirectly. For example, adsorption of agglutinins apparently leads to some kind of injury to the cell surface, the agglutinated cells becoming mechanically more fragile, final hemolysis is then brought about rapidly by complement or more slowly by mechanical trauma.<sup>24</sup> Brunius has calculated that some 30 antibody molecules would be sufficient to sensitize a red cell so that when some 6,000 molecules of complement are added, the cell hemolyzes. Sensitization or agglutination may also be brought about by non specific substances such as silicic acid, tannin, or the protein conconavalin A, here too, there is an increase in mechanical fragility, and the addition of complement also results in hemolysis. The complexity of this phenomenon is illustrated by the complexity of the composition of complement. Complement appears to be a mixture of euglobulin, two mucoproteins, and a heat stable compound containing phosphorus. All of the components of complement must attach in the proper order before hemolysis will occur.<sup>25</sup>

Another illustration of surface specificity is suggested by the phenomenon connected with a cold hemolysin.<sup>26</sup> For hemolysis of red cells to occur in cold paroxysmal hemoglobinuria, a hemolysin and components of complement are required. When the cells are chilled, the hemolysin and a component of complement are adsorbed simultaneously. Then on warming, hemolysis will occur, but only if another component of complement (a heat-labile component) is present. In one case,

if the chilling of the red cells occurred in the presence of either sulfanilamide or cyanide, hemolysis did not take place on subsequent warming. It could be shown that these reagents had not prevented the adsorption of hemolysin or complement. After dialyzing away the cyanide or sulfanilamide hemolysis occurred. In this case, the cold hemolysin and complement appear to be absorbed in the immediate vicinity of a molecule of carbonic anhydrase which is poisoned specifically by cyanide or sulfanilamide. The attachment of either of these reagents presumably at the zinc atom of the carbonic anhydrase prevented the lysis of the red cell.

A recent interesting interpretation of the ultrastructure of the ghost is that of a meshwork of tangentially arranged, long fibrous protein molecules, each being surrounded by about one hundred lipid molecules which are oriented radially around the fibrous protein.<sup>27</sup> It is not possible to decide as yet whether the ghost is balloon-like and hollow inside, or whether it contains a gel-like interior. If the stroma substance is present at all in the interior, it is probably very low in concentration. In isotonic saline, the ghost is a biconcave disc even after extraction of the lipids,<sup>28</sup> so the principal shape factors of the red cell may normally be considered to reside in the protein structure of the ghost. Waugh and Schmitt<sup>29</sup> have shown that the central region of the ghost, corresponding to the biconcavity, is thicker in protein by 30–40 Å than is the region near the edges of the ghost. In the classic work on the isoelectric point of hemoglobin, Michaelis and Takahashi<sup>30</sup> reported that hemolyzed red cells, in which excess hemoglobin was not washed away, had an agglutination optimum of pH 5.0 for all species investigated, suggesting that the isoelectric point of the stroma proteins might be at this pH. Since hemoglobin is found to leak out of a red cell immersed in a solution below pH 5.0, they hypothesized that when the pH was below 5.0 the stroma would have a positive charge, and only then would hemoglobin leak out.

**Lipids.** The lipids constitute about 0.4 per cent of the fresh weight of the human red cell and over 90 per cent of this material is present in the stroma substance. Some 33 per cent of the lipid is cephalin, 21 per cent is lecithin, 20 per cent is cholesterol, 5 per cent is made up of cholesterol esters, 3 per cent is neutral fat and 9 per cent is made up of the cerebroside.<sup>18</sup> For lyophilized human red cells Hack<sup>31</sup> reports phosphatide values per liter of red cells as: total phosphatide 3.05 Gm, cephalin 1.25 Gm, lecithin 0.92 Gm, sphingomyelin 0.88 Gm. Cephalin is the largest phospholipid fraction in the cells and the sphingomyelin concentration of the cells is twice that of the plasma. According to Parpart,<sup>32</sup> about 40–60 per cent of the lipids are bound to the stroma proteins, there being considerable variation in the different species.

Ballentine and Parpart<sup>33</sup> have found that pancreatic lipase acting on beef or rabbit erythrocytes at pH 5.0–5.5, increases the rate of penetration of ethylene glycol and glycerol, i.e., substances which are considered to enter the cell surface by way of aqueous channels. They have postulated that the lipase probably splits off one fatty acid residue per phospholipid molecule, but the fatty acids thus split off remain bound to the surface. They suggest that the phospholipid portion of the surface and the orientation of this lipid material form an architectural unit in the structure of the aqueous channels.

*Proteins* The insoluble protein constituent of the stroma, the stromatin, has properties of solubility resembling those of the albuminoids. It is rapidly digested by crude trypsin or by activated papain preparations, although the intact erythrocyte itself does not appear to be affected by crystallin trypsin.<sup>33</sup> As compared with globin, the stromatin is lower in histidine and lysine but higher in arginine. It contains 2.1 per cent histidine, 5.0 per cent arginine and 3.6 per cent lysine.<sup>34</sup>

An anti-sphering protein, which is in part responsible for the maintenance of the disk shape of the red cell, has been identified by Furchgott and Ponder,<sup>35</sup> as a fraction of serum albumin poor in carbohydrate. Red cells may be freed of this protein by adsorption of the protein on glass surfaces. Normally, at a pH above 11.3, but not below this pH, the red cell will become spherical. If, however, the antisphering protein has been removed, the red cell will become spherical at pH 9.2 or above. Addition to such a spherical cell of serum or plasma containing the antisphering protein will restore it to the disk shape, that is, the phenomenon is reversible. Ghosts which do not have the antisphering protein only sphere once, at pH 9.2, and almost immediately go spontaneously again to the disk shape, and the phenomenon is not reversible. These results explain the sphering of the red cells often observed when viewed in saline between somewhat alkaline coverslips. The quantity of this protein taken up by the spherized cells to bring them back to normal shape is sufficient to form a layer of some 50 Å at the surface. The adsorption of this protein does not appear to change the electrokinetic properties of the cell.

Calvin and co-workers<sup>36</sup> have recently reported the isolation of a lipo-protein complex 'elinin', which is said to constitute 40 per cent of the stroma substance, the remainder being 'stromatin'. The stromatin N is 12.3 and P 0.42, the elinin N is 9.1 and P 1.0. Elinin was isolated from cold, washed, human ghosts at pH 7-8 by centrifugation at 50,000 G, the stromatin remaining in solution. Elinin dissolves readily at pH 7-8 to give a milky solution with marked streaming birefringence. It contains about 40-50 per cent of lipids extractable with 3:1 alcohol. The Rh antigen was found to be solely in the elinin fraction, and the content of A and B antigens was four to five times higher in this fraction than in the stroma substance. The Rh antigen is particularly sensitive to thermal inactivation, being destroyed by exposure to 56°C for a few minutes. The A and B antigens are, in contrast, rather heat stable. An ether soluble fraction has been separated from elinin which contains a still higher content of Rh antigen.

*Blood Group Substances* A number of blood group substances in the stroma of human red blood cells are known, resulting from the allelic series, A<sub>1</sub>, A<sub>2</sub>, B and O, the allelic series M, N<sub>1</sub> and N<sub>2</sub>, and the Rh group of substances which may be an allelic series, or at least closely related, i.e., due to genes closely linked on the same chromosome.<sup>37</sup> According to Landsteiner,<sup>38</sup> the little that is known of their chemistry suggests that they may represent a new type of biologic compound. The group substances that have been studied have a high content of acetyl glucosamine, and galactose and contain amino acids in peptide linkage. The proportion of hydroxy amino acids, threonine and hydroxyproline in both group O and group A substances is higher in concentration than is found in most proteins. From the

stroma of erythrocytes the group substances may be extracted to a certain extent with alcohol but not with water, indicating that they may be present in some lipid combination in the stroma. After extraction from the stroma they appear to be water soluble.

The work of Kabat et al.<sup>39</sup> has indicated that the blood group A substances prepared from human sources such as saliva, stomach and amniotic fluid were similar in content of nitrogen, glucosamine, reducing sugar, and acetyl values. The substances A<sub>1</sub> and A<sub>2</sub> differed in optical rotation. All of the human A substance were levorotatory while the A substance from hog stomach was dextrorotatory. Landsteiner and Harte<sup>40</sup> considered that about one-fourth of the total N represented amino acid nitrogen. Morgan,<sup>41</sup> using paper chromatography, qualitatively identified at least fifteen different amino acids in the acid hydrolyzate of A substance.

The group O substance, isolated from cystic fluid by Morgan and Waddell,<sup>42</sup> contained about 43 per cent of the N as  $\alpha$  amino acid nitrogen. Some of these amino acids were suggested to be joined by glycosidic linkage to the first carbon atom of hexosamine, a linkage which is extremely susceptible to the action of dilute alkali. Studies of group A and O substances, obtained from the stomach linings of hogs, have revealed no chemical differences, except in their immunological properties. They are similar in viscosity and electrophoretic mobility at pH 7.4, both contain l-fucose, d-galactose and d-glucosamine and both also have the same nitrogen, reducing sugar, glucosamine and acetyl content.<sup>43</sup>

### III. STRUCTURE OF HEMOGLOBIN AND THE FUNCTION OF SOME OF ITS PARTS

*Hemoglobin* is the major constituent of the mature red cell, making up about 95 per cent of the dry weight of the cell. Here we have a remarkable achievement in differentiation and specialization. Not only is the hemoglobin specialized for the transport of oxygen, but it also functions indirectly to bring about the transport of CO<sub>2</sub>, without changing the pH of the blood stream. We shall now proceed to an examination of the anatomy of the hemoglobin molecule and consider some of the functions of its parts.

Hemoglobin is a protein molecule of molecular weight 68,000 (fig. 2). It is made up of a large colorless protein portion called globin. On the surface of this globin are present four small prosthetic groups of heme molecules. Two hemes are represented on the proximal surface and two other hemes are on the distal surface, both sets of hemes lying in parallel planes. These four heme molecules are colored and impart the red color to the hemoglobin molecule. The heme molecule is a metal complex consisting of an iron atom in the center of a porphyrin structure. The naturally occurring heme, which is ubiquitous in protoplasm and is present as a prosthetic group in various oxygen transporting proteins, and in a number of enzymes such as catalase, peroxidase, etc., is iron protoporphyrin 9 (fig. 3).

*Protoporphyrin 9*. This molecule consists essentially of four small pyrrole rings attached to each other through —CH methene bridges. This makes an innermost sixteen-membered ring of carbon and nitrogen atoms held together by a conjugated chain of alternating single and double bonds. Such a structure is called a resonating structure and contains  $\pi$  electrons, which appear to have free mobility along the

conjugated chain. The individual pyrrole rings also enter into this resonance, and the inner large ring and smaller pyrrole rings may be considered to be, to a limited extent, pathways for the conduction of the mobile  $\pi$  electrons. All the atoms partaking in this resonating structure lie in the same plane so that the porphyrin molecule is a flat one. The entire structure is greatly stabilized to heat and to strong acids. In addition to the stabilizing effect of the resonance on the porphyrin, the stability of the pyrrole rings is also enhanced by the presence of side chains.

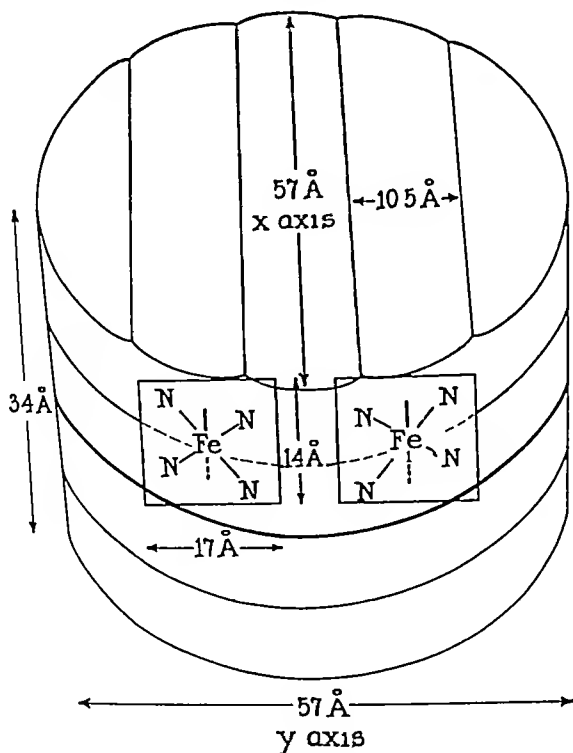


FIG. 2.—INTERPRETATION OF STRUCTURE OF HORSE HEMOGLOBIN

There are four hemes per globin molecule, two hemes are located on the proximal surface with their planes perpendicular to the upper two polypeptide layers. The other two hemes are located on the distal surface perpendicular to the lower two polypeptide layers.

In this particular porphyrin, i.e., protoporphyrin, there are three kinds of side chains, namely four methyl groups, two vinyl groups, and two propionic acid groups. Of the fifteen possible arrangements of the side chains around the circumference of the porphyrin, the natural isomer of protoporphyrin, designated as isomer #9, has the following arrangement, namely methyl, vinyl, methyl, vinyl, methyl, propionic acid, propionic acid, methyl.

Using tracer methods, Shemin and Rittenberg<sup>44</sup> have found that the nitrogens of the four pyrrole rings are derived from the N of the amino acid glycine. The  $\alpha$  C of the glycine forms an  $\alpha$  C of the pyrrole ring, but the carboxyl of glycine is



split off during some step of the synthesis.<sup>46-48</sup> Experiments with labeled acetic acid suggest that both C atoms are utilized, possibly for the synthesis of the side chains, but it is not yet clear whether acetic acid is directly incorporated.<sup>44</sup>

Evidence derived from studies of porphyrin metabolism in *Hemophilus influenzae*<sup>47</sup> suggests that the protoporphyrin ring is not formed piecemeal around an iron atom, but rather it is necessary to assume that the protoporphyrin ring is first completely formed and iron is inserted thereafter. The vinyl groups of the protoporphyrin ring appear to be essential if the organism is to be capable of inserting the iron into the protoporphyrin ring. It is not yet known what enzyme system is concerned with the process of iron insertion. Evidence also suggests that the two propionic acid side chains function in the ionized form to orient the heme and help attach the heme to the globin. The two negatively charged carboxyl groups are postulated to attach to two positively charged groups of the globin with a pK in the neighborhood of 12, possibly guanidino groups of arginine.

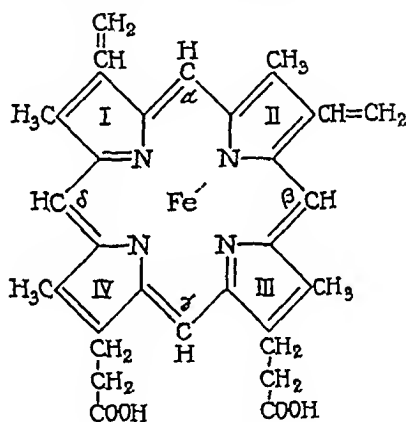


FIG. 3.—Fe PROTOPORPHYRIN

The concentration of protoporphyrin in the mature human red cells is very low being about 2.6–3.8  $\gamma$  per 100 cc. of red cells.<sup>48</sup> The red fluorescent pigment in red cells, first noted by Van den Bergh in 1928, was isolated and identified as protoporphyrin 9 by Grotepass<sup>49</sup> in 1937. As with all intermediates the content of free protoporphyrin depends on the rate of its synthesis from precursors and the rate of its removal by its combination with iron to form heme. In the developing and maturing red cell, the rise and decline in the rate of protoporphyrin synthesis appears to follow the rise and decline in the rate of hemoglobin synthesis. For example, erythroblasts contain no protoporphyrin, normoblasts are richest in it, reticulocytes contain less and the mature erythrocyte still less. In general, protoporphyrin is found to be high in cases of iron deficiency anemia (10–20 times the normal), in anemia of chronic infections (where iron does not appear to be mobilized) and in lead poisoning.<sup>48</sup> Low values have been reported in pyridoxine deficiency. London,<sup>50</sup> using labeled glycine, has recently shown that reticulocytes

induced by phenylhydrazine in the rabbit can synthesize heme in vivo. Watson, Grinstein and Hawkinson<sup>51</sup> had reported that by incubating blood in vitro for 24-48 hours, an increase in protoporphyrin was noted. In the light of the tracer experiments, this increase might now be interpreted as an indication of protoporphyrin synthesis in the incubated cells.

*Iron Protoporphyrin* <sup>9</sup> The space in the center of the porphyrin is of the correct size to accommodate an iron atom. Larger and smaller atoms can be held but they are in general (with the exception of copper) held less firmly than is iron. The iron is hexavalent,  $Fe^{2+}$ , it can bind six atoms or atom groups. In the plane of the flat porphyrin ring it links up with four N atoms. It may now bind one atom or atom group below the plane of the ring and one above the plane of the ring (fig. 2). When the bond of the iron below the plane of the ring is attached to a certain group in the globin molecule, then the remaining coordination valence above the plane of the ring can attach reversibly to oxygen and can act as an oxygen transporter.

In the bone marrow, as well as in the liver and spleen, a storage form of iron is present, called ferritin<sup>52</sup>. This is a protein which contains over 20 per cent by dry weight of iron. The protein has a molecular weight of 460,000. Attached to the surface of the protein are clusters or micelles of ferric hydroxide molecules of a special kind. The iron of this storage protein is postulated to be made available by reducing substances in the tissues which reduce the ferric iron to the ferrous form. The ferrous iron is sufficiently soluble so that it might diffuse short distances in the bone marrow, perhaps into immature red cells where hemoglobin synthesis was taking place. The protein component of ferritin is found to be depleted in response to rapid blood formation suggesting that the amino acids of ferritin might be utilized in the synthesis of proteins like globin.

The synthesis of heme may be followed by means of tracer labeled glycine.<sup>44</sup> It has been found that there is no turnover in the hemoglobin of the mature red cell. The average life span of heme and hemoglobin is the same as that of the red cell, about 120 days. By incubating duck erythrocytes with labeled glycine in vitro at 37°C, Shemin<sup>14</sup> has been able to demonstrate the incorporation of  $N_{15}$  into the hemoglobin heme. Immature cells obtained by frequent bleedings synthesize to a greater extent. London et al.<sup>60-62</sup> have found that rabbit reticulocytes in vitro can also incorporate labeled glycine into heme and that the whole blood of sickle cell anemia subjects can synthesize heme in vitro, in sickle-cell-trait without anemia no synthesis was observed.

No method has been developed to determine the free heme of the red cell and no information is available as to the relative levels of heme and globin in the developing red cells. After feeding labeled glycine, London<sup>60</sup> found a rapid early and unexpectedly high rate of excretion of labeled stercobilin. One explanation that has been suggested for this result is that heme synthesis takes place in the young red cells in great excess over that of globin synthesis and that the excess heme which is not bound to the globin is broken down and excreted in the bile.

*The Amino Acids of Globin and Certain of their Functions* The protein globin has a molecular weight of 66,000. It is a more basic protein than most of the tissue

proteins. The isoelectric point is 6.8 for ferrous hemoglobin and 6.5 for oxyhemoglobin (54). The amino acid composition of globin is not completely known. A study of the amino acids of hemoglobin of a number of mammalian species reveals that the globins are rather similar in the relative amounts of several of the different amino acids contained in them. For example, the approximate molecular ratios of tryptophane:tyrosine:arginine:histidine:lysine are 1:3:3:8:10, respectively, or, in terms of the number of molecules of these amino acids per globin, approximately

TABLE 1—*Amino Acid Analysis of Hemoglobin in per cent*  
(Modified from Cartwright<sup>55, 56, 57, 58</sup>)

Amino acid	Horse	Man	Number of amino acid residues per horse globin molecule
Leucine	15.1	17.2	76
Isoleucine	0.11	0	8
Histidine	7.7	8.0	33
Arginine	3.7	4.2	14
Lysine	8.6	—	41
Valine	9.8	10.1	64
Tryptophane	1.1	—	4
Phenylalanine	6.8	—	27
Methionine	0.75	1.2	3
Cystine	0.85	1.2	2
Threonine	6.8	6.8	38
Tyrosine	3.0	—	12
Alanine	8.9	9.9	61
Glycine	5.6	—	50
Serine	5.3	—	33
Proline	2.0	—	11
Hydroxyproline	0.0	—	0
Aspartic	10.3	—	51
Glutamic	8.5	—	39
Amide N in % of total N	4.58	—	48
Total S	0.4	0.6	
Total N	16.4	16.6	
Total Fe	0.335	0.335	4

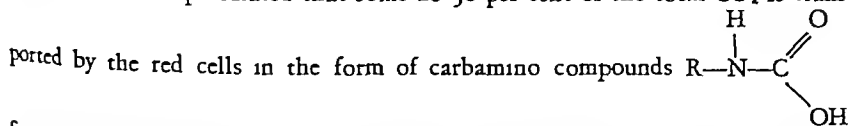
as 4:12:14:33:41. The relative high content of arginine, histidine and lysine residues are in part responsible for the high isoelectric point of globin. Because of this high isoelectric point the affinity for acidic dyes such as eosin is greatly enhanced. Since more data is available on horse hemoglobin, this protein will be considered in an analysis of hemoglobin structure.

Some functions may be postulated for several of the constituent amino acids of globin. Relative to other proteins, globin is one of the richest in histidine content, there being about 33 histidines per globin molecule. The histidines may be postulated to have three functions. The most important function would appear to be that one of the imidazole nitrogens furnishes the seat of attachment for one of the coordination valences of the iron of heme, thus making possible the reversible oxy-

genation of hemoglobin (*vide infra*). The evidence for this iron link is indirect. Four histidine residues would be involved, one for each of the four hemes on the globin. It is further postulated that the second nitrogen of these same imidazole groups would be involved in the change of acidity with change in oxygenation of the hemoglobin. This Bohr effect makes possible the transport of  $\text{CO}_2$  as bicarbonate ion. The third function of histidine in hemoglobin is due to the fact that the histidine residues serve as excellent buffers, since the  $\text{pK}$  of the imidazole nitrogens lies in physiological pH range. Some 29 histidine residues may function in this regard. According to Wyman, between pH 5.5 and 8.8 there are titrated 31 groups in all, most of which probably are in the histidines. This buffering capacity would become important in cases where much lactic acid escaped into the blood stream, as in severe exercise.

The lysine  $\epsilon$ -amino groups and the arginine-guanidino groups make up a total of 55 positively charged groups. Eight of these positively charged groups are postulated to be combined with eight propionic acid side chains of the four hemes. Porter and Sanger<sup>59</sup> have found that the reagent, fluoro dinitrobenzene, determines all of the  $\epsilon$ -amino groups of lysine in the presence or absence of the hemes. This suggests that all the  $\epsilon$ -amino groups are free, that they lie on the surface of the globin, and that the propionic acid groups of heme are most probably attached to the guanidino groups of arginine rather than to the  $\epsilon$ -amino groups of lysine. Including the six positively charged amino groups at the ends of the polypeptide chains this would make a total of 53 free positively charged groups at pH 7, exclusive of the imidazole residues. The ionized carboxyl groups, of glutamic and aspartic acid together, total 43 groups after correction is made for amide nitrogen. In addition there are six carboxyl groups at the ends of polypeptide chains, thus giving a total of 49 negatively charged groups, these probably play an insignificant role in buffering.

Roughton<sup>60</sup> has postulated that some 20-30 per cent of the total  $\text{CO}_2$  is trans-



Since carbamate formation is decreased in oxyhemoglobin as compared with hemoglobin, Roughton has suggested that the attachment of the  $\text{O}_2$  in some way may interfere sterically with the formation of carbamate. Carbamic acids have a  $\text{pK}$  of about 5.8. Only uncharged  $\alpha$  amino groups combine with  $\text{CO}_2$  to form carbamino compounds. In horse globin six free amino groups are present (in the human, 5), namely, the amino groups of valine at the ends of the peptide chains.<sup>59</sup> Such amino groups would have a  $\text{pK}$  around 9 and would be present at body pH in the form of  $\text{R}-\text{NH}_3^+$ . Since  $\text{CO}_2$  combines with the  $\text{R}-\text{NH}_2$  group, it would be necessary to postulate a shift of the valine amino group by some 2 pH units to the acid side to account for significant  $\text{CO}_2$  transport as carbamino compound. Wyman has calculated that transport of 10 per cent of  $\text{CO}_2$  in the respiratory cycle could be due to carbamate.<sup>61</sup> The assumptions and postulates on carbamate have been discussed in detail more recently by Wyman.<sup>61</sup>

It is interesting to note a possible structural function of another amino acid, proline. Pauling<sup>67</sup> has suggested that proline, because of its geometry might serve as a 90-degree bend or elbow-joint in the polypeptide chain.

*Hemoglobin Structure* Intensive x-ray analyses of horse ferric hemoglobin by Boyes-Watson, Davidson and Perutz<sup>68</sup> have led to a clearer concept of the structure of the hemoglobin molecule. It has a molecular weight of 68,000 and its dimensions, calculated on the basis of a cylindrical structure, with convex ends, are  $57 \times 57 \text{ \AA}$  by  $34 \text{ \AA}$  high (fig. 2.) The assumption of a cylindrical structure for the volume of the globin is a mathematically convenient one. It is not a unique solution for the data, however, and should be considered only as an approximation to the actual shape.

One interpretation of the globin structure is that in the  $34 \text{ \AA}$  dimension the globin consists of four parallel monolayers of polypeptides, each about  $8\frac{1}{2} \text{ \AA}$  thick. This  $8\frac{1}{2} \text{ \AA}$  dimension corresponds to the average thickness of a monolayer of a protein, including its side chains, when completely spread out on a Langmuir trough. The polypeptide layers contain polypeptide chains or rods running the length of the molecule along the x direction.<sup>69</sup> The polypeptide chain is not fully extended. Along its length there appear to be regularly spaced small folds some  $5 \text{ \AA}$  apart, the folds being equivalent to a distance of two amino acid residues along the chain. This kind of fold is similar to that described by Astbury as the contracted or alpha-keratin type. According to this interpretation the wrinkled polypeptide chain running along the x direction would be a roughly cylindrical elongated rod with an average diameter of  $10.5 \text{ \AA}$ . Along the y direction, i.e., in a polypeptide layer of the globin, one could fit about 5 such rods, or the 4 polypeptide layers could contain a total of 20 such rods. The studies of Porter and Sanger<sup>70</sup> reveal only six free amino groups suggesting that there are only six polypeptide chains in horse globin. However, as yet it is not possible to rule out cyclic chains, nor chains branching off from some carboxyl groups of glutamic or aspartic acids.

It can be determined from optic dichroism that the four hemes, which are flat or planar molecules, lie parallel to each other in the protein crystal. Because the hemes can be oxidized to the ferric state with relatively large molecules such as ferricyanide, and because the hemes may be removed from the globin in a somewhat reversible manner,<sup>71</sup> it is reasonable to consider that the hemes are on the surface of the globin. In addition, the stability of the heme globin combination makes it probable that Van der Waals forces occur between the resonating ring and the globin surface and it is reasonable to assume the arrangement of heme and globin to be such that the plane of the flat heme molecule lies on an area of the globin surface which is planar. From x-ray data, it is known that the plane of the heme is perpendicular to the x axis of the globin. The hemes, therefore, lie perpendicular to the ends of the polypeptide rods. Studies of the protein in solutions of high urea or salt concentration suggest that the protein is split into two identical halves, each half being made up of two polypeptide layers. Since a plane of symmetry is present in the hemoglobin molecule, each half of the molecule must be structurally and chemically identical.<sup>72</sup> These facts suggest that a pair of hemes belong to the upper half of the molecule and a pair of hemes belong to

the lower half Wyman<sup>54</sup> interprets the hyperbolic oxygenation curve of hemoglobin as indicating that there is a strong interaction between hemes belonging to the same pair and little interaction between hemes belonging to different pairs. On this basis, the hemes belonging to the same pair are placed close together, one pair of hemes being represented on the proximal surface of the molecule lying perpendicular to the two upper polypeptide layers (fig. 2), the other pair of hemes being on the distal surface of the molecule and lying perpendicular to the two lower polypeptide layers. It is interesting to consider that the attachment of the hemes to these rods of polypeptides binds the rods together and stabilizes an otherwise highly unstable protein molecule.

The density of the protein indicates that the polypeptide chains in the layers, and the layers themselves, are packed very closely. The water associated with the protein is probably present as one or perhaps two monolayers surrounding the protein molecule.

*Effects of Interaction of Heme-iron and Globin* The iron atom in Fe protoporphyrin 9, 1c, heme, has the ability to bind six groups, that is, it has a coordination valence of 6. It binds the four pyrrole nitrogens in the plane of the ring and is able to bind a group below the plane of the ring and a group above the plane of the ring. In hemoglobin, it is postulated<sup>55, 54</sup> that the iron is attached to a nitrogen of the imidazole group of histidine. When this attachment occurs, and when the iron is in the ferrous, that is, the reduced state, then the molecule of O<sub>2</sub> can be attached reversibly at the sixth coordination place of the iron. Wyman has shown that from the effect of temperature on the titration curve of oxyhemoglobin a heat of dissociation of 6,200 calories per mol can be calculated, a value expected for ionizable groups like the nitrogen of imidazole. The effect of change of pH on the heat of oxygenation also gives a value compatible with such compounds. The effect of pH and temperature on the form of the O<sub>2</sub> equilibrium curve is interpreted by Wyman as evidence that all the hemes are attached to identical local configurations on the globin.

The linkage of the iron of the heme to the protein is an all-important one. It endows the complex of heme and globin with the peculiar property of permitting the sixth coordination link to be reversibly held by an oxygen molecule. Ferrous heme, free in solution, is rapidly oxidized by O<sub>2</sub> to the state of ferric heme. This oxidation does not occur when the heme is attached to globin. The binding of the ferrous iron of heme to globin permits the addition of O<sub>2</sub> but the O<sub>2</sub> here is stabilized. The O<sub>2</sub> cannot act as an oxidizing agent, that is, it cannot accept an electron. If one measures ferrous hemoglobin magnetically it is found that the iron has four unpaired electrons in its 3d shell. The O<sub>2</sub> molecule is actually a biradical and so possesses two unpaired electrons. Now when O<sub>2</sub> unites with ferrous hemoglobin, a profound change in the magnetic susceptibility is observed.<sup>56</sup> The resulting compound, oxyhemoglobin, is diamagnetic. This means that all the unpaired electrons of the iron and of the O<sub>2</sub> have paired. And it is perhaps this change which is significant in preventing the ferrous globin from being oxidized to the ferric state (i.e., to methemoglobin).

Combination of hemoglobin with oxygen is a much more rapid process than is

its dissociation. The rapidity with which oxygen may be combined or removed is brought out by the following data and calculations. The velocity of combination of oxygen with hemoglobin to form oxyhemoglobin and the dissociation of oxyhemoglobin to form hemoglobin and oxygen have been measured in sheep blood at 20 C by Hartridge and Roughton.<sup>67</sup> Considering that each heme of hemoglobin is independent of other hemes, the first order velocity constant for the dissociation of the heme-O<sub>2</sub> complex into heme + O<sub>2</sub> is 20 per second, that is, it would require 0.035 seconds for half of the oxyhemoglobin to dissociate. For the combination of the heme of hemoglobin with O<sub>2</sub>, the second order velocity constant is 3,000 per second per millimole liter, that is, assuming 9.4 as the millimolar concentration of heme in the blood and an equimillimolar concentration of O<sub>2</sub>, then 50 per cent of hemoglobin would be combined with O<sub>2</sub> in  $3.5 \times 10^{-3}$  seconds. From this we see that the velocity of combination of O<sub>2</sub> with heme to the 50 per cent point is 1000 times as great as the velocity of dissociation of oxyhemoglobin to the 50 per cent point.

It is interesting to compare these figures of oxygenation with those in which CO combines with hemoglobin. For the dissociation of carbon monoxide hemoglobin the first order velocity constant is 0.01 per second. For its formation, the second order velocity constant is 250 per second per millimole liter. The CO complex therefore is formed at only one-tenth the velocity of formation of the O complex. For a dissociation of 50 per cent of carbon monoxide hemoglobin, 69 seconds would be required. For the formation of 50 per cent of carbon monoxide hemoglobin, assuming the millimolar concentration of heme in the blood and an equimillimolar concentration of CO,  $4.2 \times 10^{-4}$  seconds would be required. Thus the velocity of combination of CO to the 50 per cent point would be 160,000 times as great as its dissociation to the 50 per cent point.

The linkage of the iron of heme to the globin is important in another respect, namely, in the so-called isohydric transport of CO<sub>2</sub> in the blood stream. From the data of Wyman it appears that in oxyhemoglobin a group with a pK of 6.8 is present, and that when O<sub>2</sub> is removed the pK of this group changes to 7.8. In other words, at the pH of the blood, when O<sub>2</sub> adds to hemoglobin a proton (H<sup>+</sup>) tends to dissociate off. When O<sub>2</sub> comes off the oxyhemoglobin, a proton tends to go back on (fig. 4). This effect, of hemoglobin becoming more acidic on oxygenation, is called the Bohr effect.

The group which undergoes this change on oxygenation has been suggested by Coryell and Pauling<sup>68</sup> and by Wyman<sup>64</sup> to be the nitrogen of the heme linked imidazole group. Pictured in more detail (fig. 4) one might consider that the resonating ring of the porphyrin is coupled through the iron to the resonating imidazole ring. Then the addition of O<sub>2</sub> to hemoglobin would cause a slight displacement of the resonating electrons toward the O<sub>2</sub>, this would make the imidazole nitrogen a little more positive, thus decreasing the binding of this nitrogen for its H<sup>+</sup>, and the H<sup>+</sup> would tend to dissociate off more readily (i.e., the pH of the nitrogen would shift from 7.8 to 6.8). This effect is as though O<sub>2</sub> itself were an acid and enabled the blood to carry 0.7 mol of H<sub>2</sub>CO<sub>3</sub> as bicarbonate ion per mol

of  $O_2$  exchanged, or some 60 per cent of the total  $CO_2$ , without any pH alteration<sup>66</sup>

In figure 4 some of the events of  $CO_2$  transport in connection with hemoglobin have been summarized schematically. Here a heme group is shown sitting on the globin surface, the iron coordinating with the four pyrrole ring nitrogens, a fifth coordination link being connected with a nitrogen of an imidazole ring of histidine in the peptide chain of the globin. The sixth coordination link now may combine with  $O_2$  reversibly

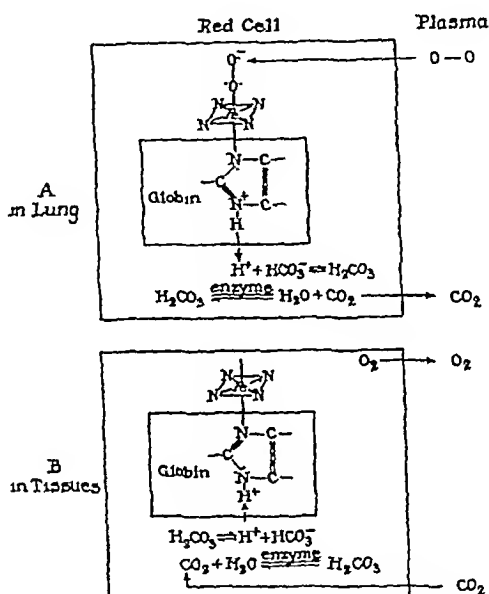


FIG. 4—SCHEME REPRESENTING THE EFFECT OF OXYGENATION OF HEMOGLOBIN ON THE TRANSPORT OF  $CO_2$  AS BICARBONATE ION

The pathway of  $CO_2$  and  $O_2$  molecules in the blood stream is shown on the diagram. When blood comes to the lungs (fig. 4-A),  $O_2$  adds to the 6th coordination link, a rearrangement of electrons occurs, bringing about the dissociation of a proton,  $H^+$ , from the imidazole-N. The proton very rapidly combines with  $HCO_3^-$  to form  $H_2CO_3$  (ionization reactions being extremely rapid). Conversion of  $H_2CO_3$  to  $H_2O$  and  $CO_2$ , i.e., dehydration-hydration reaction, is relatively slow. The attainment of equilibrium of this reaction is catalyzed by the zinc protein enzyme, carbonic anhydrase. The excess  $CO_2$  produced diffuses into the plasma and out through the lungs.

The blood then flows toward the tissues (fig. 4-B). Here the  $CO_2$  moves into the red cells to be hydrated to  $H_2CO_3$  by way of the carbonic anhydrase enzyme. The  $H_2CO_3$  dissociates to  $H^+$  and  $HCO_3^-$ . At the same time that  $O_2$  comes off, the ferrous hemoglobin now accepts the  $H^+$ . In connection with all of these events



there occur shifts in  $\text{Cl}^-$ ,  $\text{HCO}_3^-$  and  $\text{H}_2\text{O}$ , to take care of Donnan ion effects and osmotic changes,<sup>68</sup> but these cannot be considered here

According to the hypothesis of the heme-linked imidazole group, one might expect all  $\text{O}_2$ -transporting heme proteins to show a significant Bohr effect. This Bohr effect has been observed for the circulating mammalian and most invertebrate hemoglobins.\* However, a number of exceptions have been noted. The Bohr effect for muscle hemoglobin is so small that it may be within experimental error, and no Bohr effect has been observed in *Gastrophilus*<sup>69</sup> or *Urechis* hemoglobins. Whether a modified imidazole must be postulated in these latter cases, or some other group or effect, is not known. Another objection that has been raised to the heme-linked imidazole hypothesis is that the addition of imidazole compounds directly to heme gives no evidence for a stable oxygenation complex, but the difficulty may be that the existence of such a complex might require a stable spatial molecular arrangement.

*Other Effects of Interaction of Amino Acids Adjacent to the Hemes* When the sharp  $\alpha$  bands of  $\text{O}_2$  and CO hemoglobins are measured with a Hartridge reversion spectroscopy for various vertebrate and invertebrate species, slight differences are noted in the positions of the bands. The position of the bands for a particular species is highly constant and indeed is a delicate test to support the idea of the invariable character of a specific protein in a given species. Among various species the difference between the wave length of the  $\alpha$  band of  $\text{O}_2$  and CO hemoglobin, called the span, may vary by as much as  $\pm 40 \text{ \AA}$ .<sup>70</sup> These displacements of band positions are considered to be due to differences in the kinds of amino acid groups adjacent to the heme and their spatial distribution around the heme. Another expression of differences in behaviour of different hemoglobins is the relative affinity of  $\text{O}_2$  as compared to CO for the hemoglobin of a particular species.<sup>71</sup>

If the position of the amino acids surrounding the heme may affect the positions of the absorption bands, one might consider that the change on oxygenation of hemoglobin might also affect some of these neighboring amino acids. For example, on oxygenation of horse hemoglobin there appears to be evidence for a group changing from  $\text{pK } 5.25$  to  $\text{pK } 5.75$ .<sup>54</sup> This group has been postulated to be an imidazole group whose proximity to the heme, rather than direct linkage, might affect its ionization. One might also explain some of the changes in carbamate concentration as affected by oxygenation, by postulating valine amino groups at the ends of the peptide chains to be located adjacent to the hemes. Eventually too, the variation in affinity of various hemoglobins for  $\text{O}_2$  with changes in  $\text{O}_2$  tension, may find their explanation on the basis of structural effects of amino acids of globin adjacent to the heme.

*The Form of Hemoglobin in the Erythrocyte* The erythrocyte contains some 30 per cent, by wet weight, of hemoglobin. This concentration of hemoglobin is so high that one might expect to observe properties of hemoglobin which deviate from hemoglobin in dilute solution. Ponder<sup>18</sup> has calculated that if hemoglobin mole

\* As noted by Redfield<sup>127</sup> there is a considerable variation in the degree of the Bohr effect among various species.

cules were arranged in hexagonal packing there would only be enough water to form a layer of 10 Å around each hemoglobin. On the average, the distance apart between two hemoglobin molecules at closest approach in the red cell, as determined by x-ray diffraction, is of the order of magnitude of two water molecules.

When the oxyhemoglobin formed from ferrous hemoglobin is plotted against the tension of  $O_2$ , it is found that the resulting  $O_2$  affinity curve is sigmoid in shape.\* For muscle hemoglobin containing one heme per molecule the curve is a rectangular hyperbola. It has therefore been postulated that the sigmoid curve of red cell hemoglobin is due to interactions of the hemes. In horse hemoglobin the hemes appear to lie on the globin in pairs (fig. 2). Wyman believes there is strong interaction between hemes of the same pair such as to make the first  $O_2$  attach to hemoglobin less readily and the next  $O_2$  attach more readily. In support of this idea of the interaction of hemes of the same pair on each other, is suggested the fact that in strong urea solutions horse hemoglobin splits into two equal halves each containing a pair of hemes, and the sigmoid shape of the  $O_2$  equilibrium curve of these halves of molecules is largely maintained. In addition to this interaction, there appears to be an interaction between hemes of different pairs, probably hemes belonging to different ferrous hemoglobin molecules, (rather than oxyhemoglobin molecules), and this interaction may be influenced in part by dilution. It is not yet clear, however, to what extent the sigmoid curve is influenced by close approach of hemes of different molecules, by splitting of the molecules on dilution, by effects of various salts, nor is it clear how the hemes interact to affect the  $O_2$  affinity. Human hemoglobin is said to be only slightly dissociated into smaller molecules on dilution and yet shows a significant change in the  $O_2$  tension curve.

Hill and Wolvekamp<sup>72</sup> report the following interesting experiment in connection with the sigmoid curve. They found that by diluting the concentrated human hemoglobin of the red cells five times, the  $O_2$  tension for half saturation of the hemoglobin with  $O_2$  dropped from 9.8 mm  $O_2$  down to 3.1 mm  $O_2$ , and was not decreased further even on greater dilution. Seeking for an explanation of this behavior these authors discovered that a substance could be obtained by dialyzing horse corpuscles which, when added to a dilute solution of hemoglobin, shifted the  $O_2$  tension curve towards that found for more concentrated hemoglobin solutions. The substance in the dialysate was destroyed by boiling and became inactive after four days in the cold. Perhaps it is this substance which may account in part for the  $O_2$  tension curve of the red cell.

The unexpectedly low quantum yields when horse CO-hemoglobin is dissociated by light suggest the possibility of some kind of interaction in the hemoglobin molecule as a unit. Warburg<sup>129</sup> reports that only one CO is split off for 4 light quanta absorbed, perhaps indicating that the binding of one of the four CO-hemes is looser than the others. On splitting hemoglobin into two equal halves (in 2.6 M NaCl at pH 7.6), one CO is split off for 2 light quanta absorbed. In muscle

\* This is true in general for mammalian erythrocyte hemoglobins. However in the blood of fishes the sigmoid-shaped curve may be scarcely observed or may be lacking. The peculiar curves for duck and pigeon hemoglobins where a slow proportionate increase of oxyhemoglobin occurs with increasing  $O_2$  tension have yet to be explained.<sup>127</sup>

CO-hemoglobin, where there is only one heme per protein molecule of molecular weight 16,500, one CO is split off for every light quantum absorbed

Another problem in connection with the very high concentration of hemoglobin in the red cells is, why does not the hemoglobin normally crystallize out within the cell? For example, when guinea pig red cells are laked with water, crystals of oxyhemoglobin appear almost immediately. These crystals are formed in a dilute solution as compared to the concentrated solution in the red cell where crystals are not observed. Not only would crystallization in the red cell be harmful by causing mechanical injury, but crystallization would also be deleterious since it has been found that oxyhemoglobin in the crystalline state holds on to its  $O_2$  so tenaciously that it could not serve for reversible  $O_2$  transport.<sup>73</sup> In sickle cells, the formation of the sickle occurs when the hemoglobin is deprived of  $O_2$ . It is interesting that human ferrous hemoglobin, unlike guinea pig hemoglobin is said to be less soluble than oxyhemoglobin, suggesting that the phenomenon of sickling might be related to incipient crystallization of the ferrous hemoglobin.

*Fetal Hemoglobin* Recent evidence has shown that a fetal hemoglobin is produced whose properties differ from those of normal adult hemoglobin. It was Barcroft who first called attention to the fact that differences existed between fetal and adult hemoglobins. Brinkman and Jonxis<sup>74</sup> showed that below the age of three in humans there was a hemoglobin which was labile to alkali, and that after this period the hemoglobin changed over to a form that was alkali stable. Wyman et al.<sup>75</sup> later found that the CO hemoglobin of fetal cow blood was more than six times as soluble in strong phosphate buffer at pH 6.8 than was adult cow blood. Vickery<sup>76</sup> analyzed these hemoglobins and found that fetal bovine hemoglobin contained  $6.43 \pm 0.04$  per cent histidine whereas adult bovine hemoglobin contained  $6.81 \pm 0.05$  per cent histidine, indicating that the change is a quantitative one and not merely a change in the spatial arrangement. In addition, Hill and Wolvekamp<sup>77</sup> have shown that fetal hemoglobin, like muscle hemoglobin, has a higher affinity for  $O_2$  than has adult hemoglobin, that is,  $O_2$  is removed from these hemoglobins at lower oxygen tensions than from adult hemoglobin. The conclusion that there are two distinct substances is thus supported. A reasonable interpretation is that the fetal hemoglobin may be produced in organs other than the bone marrow, e.g., such as the liver, and that only the adult hemoglobin is produced by the bone marrow.

#### IV PROTEINS OF THE RED BLOOD CELL

Besides the hemoglobin, the stroma proteins, and the protein enzymes of the glycolytic system (which will be considered in another section), a number of other proteins have been found in the erythrocyte.

*Metal Containing Proteins* There is only one iron containing protein in the red cell besides hemoglobin that is known, i.e., catalase. The cytochromes and cytochrome oxidase appear to be lacking. In consequence, the  $O_2$  utilization of the red cells is very small.

*Catalase* content of erythrocytes appears to be relatively high. It appears to have properties probably identical with the catalase of liver. The protein has a molecular

weight of 325,000 and contains four hemes per protein molecule.<sup>77</sup> One obvious function of catalase is the protective one, i.e., of destroying  $\text{H}_2\text{O}_2$  which might be produced in the red cells. The catalase may also have a peroxidase activity of low order and, in the presence of very small amounts of  $\text{H}_2\text{O}_2$ , it may oxidize the lower alcohols to aldehydes, the aldehydes to acids, and formic acid to  $\text{CO}_2 + \text{H}_2\text{O}$ .<sup>78</sup>

*Carbonic anhydrase* is a zinc protein which, according to Scott<sup>79</sup> contains 0.2 per cent of zinc. It has a molecular weight of 30,000 and an isoelectric point of 5.3. It was first postulated to exist by Henriques who calculated that the spontaneous decomposition of  $\text{H}_2\text{CO}_3$  is too slow a reaction to liberate  $\text{CO}_2$  from the blood stream. Meldrum and Roughton demonstrated its existence in 1932, and it was crystallized from the red cells by Keilin and Mann<sup>80</sup> in 1940. The enzyme is a hydrolytic one catalyzing the reaction  $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3$ . There is sufficient carbonic anhydrase in the red cells to accelerate this reaction about 1,500 times at 38°C and this is about ten times the required acceleration. All the zinc in the red cells is accounted for as a component of carbonic anhydrase. The enzyme is inhibited by low concentrations of cyanide and sulfanilamide.

*Hemocuprein* is a cupric-containing protein. It is bluish in color, contains 0.34 per cent Cu, has a molecular weight of 35,000 with two copper atoms per protein molecule. It was isolated by Mann and Keilin in 1938<sup>81</sup> from red cells. Its function is not known. The copper can be reduced but cannot be oxidized back to its original color. The anemia of copper deficiency, like that of iron deficiency, is microcytic and hypochromic. The possibility that a copper compound catalyzes the formation of cytochrome oxidase or is a component of the cytochrome oxidase system has not yet been eliminated.<sup>82</sup>

*Phosphomonoesterase*. There appear to be both an  $\alpha$  and a  $\beta$  glycerophosphatase in human red cells according to Paget and Vitter.<sup>83</sup>

*Choline Esterase*. Human red cells contain a potent true choline esterase, i.e., specifically inhibited by  $\beta, \beta'$  dichlorodiethyl-N-methylamine or by caffeine.<sup>84</sup> The choline esterase of human erythrocytes and of brain are very similar, if not identical, but both differ considerably from the choline esterase of human plasma. According to Mentha et al.,<sup>85</sup> the esterase can be extracted from chilled red cells at pH 8.3 with little hemolysis. This would suggest that the choline esterase may be at or near the cell surface.

Stern and coworkers<sup>86</sup> have separated two other protein components from the erythrocyte by electrophoresis, protein a probably being related to stomatin, protein b not being identified as yet. In the course of preparation of stomatin, a globulin was also found in the supernatant fluid. Elinin and the carbohydrate-poor albumin anti-sphering protein have been discussed in a previous section.

## V WATER SOLUBLE CONSTITUENTS OTHER THAN PROTEINS

All of the glutathione (GSH) of the blood is confined to the red cells where it may undergo rapid oxidation and reduction. It is suggested that together with ascorbic acid, it may play a minor role in the reduction of ferric hemoglobin to ferrous hemoglobin. Thionine, the betaine of thiol histidine, is also confined to

the red cells Porter and Franke<sup>88</sup> consider that this substance may all have been derived from the food Christensen and Lynch<sup>89</sup> believe that the concentrations of  $\alpha$  amino acids and peptide compounds (exclusive of GSH) are rather evenly distributed between the cells and the plasma

TABLE 2.—Concentration of a Number of Substances Found in Human Erythrocytes

Compounds	Concentration in mg./100 cc. red cells	Reference No.
Urea		
Creatine	14	87
Creatinine	3.1	87
Amino acids	0.7	87
Peptides (not GSH)	30	89
Glutathione	13	89
Thionine (ergothioneine)	70	87
Glucose	15	88
Total reducing substances calculated as glucose	74	15
	114	15
ATP + ADP		
DPN	50	93
TPN	10	92
Flavine dinucleotide	1.2	92
	0.075	98
Total NPN		
Nucleotide N	44	87
Amino acid N	13	87
	7.4	87
Inorganic P	2	104
Organic acid soluble P	55	104
ATP P	13.5	104
2,3 Diphosphoglycerate P	28	104
Hexose mono+ diphosphate P	15	104
Neutral S	6	106
Inorganic sulfate S	0.04	106
Ethereal sulfate-S	0.04	106
Na <sup>+</sup>	45	15
K <sup>+</sup>	420	15
Mg <sup>++</sup>	3.0	15
HCO <sub>3</sub> <sup>-</sup>	100	15
Cl <sup>-</sup>	180	15

The adenine nucleotides average about 65 mg./100 cc. of red cells, consisting mostly of adenosine triphosphate (A T P), adenosine diphosphate (A D P) and diphosphopyridine adenine dinucleotide (DPN) or coenzyme I. Smaller amounts of a triphosphopyridine adenine dinucleotide (TPN) or coenzyme II, and still smaller amounts of flavine adenine dinucleotide are also present.

In human red cells under certain conditions the ribose of adenylic acid appears

to be converted to triose phosphate, suggesting that ribose is split into a triose and a two-carbon compound.<sup>90</sup> Racker<sup>91</sup> has recently purified an enzyme from bacterial extracts which also converts ribose 5- $\text{PO}_4$  to triose phosphate.

Approximately 90 per cent of the total blood nicotinic acid is in the corpuscles, and all of the nicotinic coenzymes. The coenzymes are rapidly destroyed when the red cell undergoes hemolysis,<sup>92</sup> indicating that in the intact red cell the coenzymes are probably being broken down and built up at an appreciable rate. Handler and Kohn<sup>93</sup> found that DPN was hydrolyzed at the link between the nicotinamide and the ribose and that this hydrolysis was inhibited by nicotinamide but not by nicotinic acid. Nicotinamide, however, cannot be used directly for DPN synthesis, although nicotinic acid itself is used directly for this synthesis in the red cell. Both nicotinic acid and its amide are equally permeable to the red cell. In vivo, feeding of nicotinamide raised the coenzyme by 25-40 per cent in the circulating cells, feeding of nicotinic acid raised it, however, from 90-300 per cent. (The V factor test for *Hemophilus influenzae* growth<sup>94</sup> was used in their determinations, nicotinamide nucleoside was about one-third as active as DPN.) The enzyme synthesizing DPN is not the same as the hydrolyzing enzyme. Gutman et al.<sup>95</sup> showed that in hemolyzed red cells hexose diphosphate was more readily utilized for the reduction of ferric hemoglobin when nicotinamide was added to suppress DPN hydrolysis. In nicotinic acid deficiency an anemia results leading to degenerate anitosis and premature ripening of the erythrocytes. On feeding nicotinic acid an increase in red cells and in hemoglobin is observed. Evidently the nicotinic coenzymes are necessary even in the immature nucleated erythrocytes.<sup>96, 97</sup>

The red cells can couple ribose to the nicotinic nitrogen to form nucleoside and then phosphorylate with ATP to form nucleotide. Among the specific effects of pyridoxine deficiency leading to a hypochromic anemia in the rat, is the inability to convert tryptophane to nicotinic acid so that the red cells are low in DPN.<sup>98</sup>

No estimates of total alloxazine-containing compounds (the flavines) are available but, compared to the nicotinamide-containing compounds, they appear to be in very low concentration in the red cells. In human red cells, Klein and Kohn<sup>98</sup> found a concentration of flavine adenine dinucleotide of 0.075 mg/100 cc cells. In red cells, the flavine dinucleotide is about 300 times lower on a wet weight basis than in heart or kidney tissue and is 100 times lower than the nicotinamide coenzymes. In the red cell no d-amino acid oxidase activity, for which the flavine dinucleotide is a prosthetic group, was observed.<sup>100</sup> The experiments of Gibson<sup>101</sup> suggest that a diaphorase-like enzyme (which contains the flavine dinucleotide prosthetic group) may be present in the red cell. Electrons then may be transferred rapidly from reduced DPN through diaphorase, through some intermediate carrier, finally to ferric hemoglobin, to reduce the latter to functional ferrous hemoglobin. The addition of riboflavin (alloxazine ribose) to red cells in vivo or in vitro increased the dinucleotide content about 25 per cent after several hours. Alloxazine itself did not increase the dinucleotide content since ribose was unable to couple with it in the red cell.<sup>98</sup>

The phosphate in the red cells is predominantly in the form of organic acid-soluble P. The concentration of inorganic phosphate is here much less than in the

plasma and its value depends on the rate of synthesis and decomposition of the organic esters rather than on the factors governing the Donnan equilibrium. While the rate of penetration of inorganic phosphate into the red cell is slow as compared to other anions,<sup>107</sup> its incorporation into organic phosphate is very rapid.

In most mammalian bloods the concentration of organic acid soluble P varies between 50-100 mg/100 cc red cells with usually one half being 2,3-diphosphoglycerate. This compound differs from the labile, 1,3-diphosphoglycerate, the latter being the normal intermediate in the glycolytic scheme.

Whether the stable diphosphoglycerate is converted to the labile intermediate by way of a mutase, or is brought into the glycolytic scheme by some other means, is not yet known. Rapoport and Guest<sup>102, 105</sup> have suggested that 2,3-diphosphoglycerate might serve in adjusting the anion equivalency in the red cells to changes in the concentration of diffusible electrolytes in some pathological conditions since the concentration of this compound may change rapidly within relatively wide limits.

In reticulocytes, the ATP is 2-3 times higher than in the mature red cell. Nucleoprotein appears also to be present in reticulocytes but not in the mature cells.

It is interesting to note that the concentration of acid soluble P is higher in the nucleated erythrocytes of birds than in mammals, being 90-135 mg/100 cc. red cells with a large proportion of phytic acid-P (50-87 mg/100 cc) and no phosphoglycerate, if the cells of a species contained phytic acid, then it was found that the enzyme phytase was also present in the red cells of this species, otherwise phytase was absent.<sup>104, 108</sup>

The minerals constitute around 0.6 per cent of the wet weight of the cell. In the human red cell there is six times as much  $K^+$  as  $Na^+$ , and  $Cl^-$  is about half that of  $K^+$ .  $Mg^{++}$  is also present in small amounts and is known to serve as a catalyst for some of the steps in the glycolytic scheme. The  $Mg^{++}$  and  $K^+$  contents of reticulocytes are higher than in the mature red cell.

## VI. METABOLIC SYSTEMS OF THE MATURE ERYTHROCYTE

The average life of a circulating erythrocyte is 120 days. Attempts to maintain these cells in vitro at 37°C for even a week show that marked deterioration of these cells takes place. There is liberation of ammonia and exchange of  $K^+$  for  $Na^+$  across the cell membrane soon after the blood is drawn and concomitantly glucose disappears and lactic acid accumulates, ATP decreases, there is also a decrease of the total organic acid-soluble-P with an increase in the concentration of inorganic P, and methemoglobin is gradually formed at a faster rate than it is reduced, the cells turning brownish in color. At the same time the physical structure of the cell is affected, the change being associated with an increase in osmotic fragility (i.e., greater hemolysis in hypotonic solutions compared with the normal), increased thickness, crenation, etc.<sup>107, 108</sup> A loss of the specific polysaccharide antigens *a* and *b* also occurs.<sup>109</sup>

For the maintenance of the integrity of the red cell in vivo, glucose is undoubtedly the main energy source. However, it would appear that other factors of the plasma, continuously being generated by the tissues of the body, must also take

part. The biochemistry of some of these processes for the maintenance of the red blood cell are already apparent and one of these, the mechanism by which ferrous hemoglobin is kept in a functional condition for oxygen transport will be discussed. However, such important processes as the slow but continuous accumulation of  $K^+$  into the red blood cell against a concentration gradient of  $K^{+110}$  and the preservation of selective permeability of the cell membrane will not be considered here because their link with biochemical events is still not clear.

*Spontaneous Formation of Ferric Hemoglobin (Methemoglobin)* In normal individuals the ferric hemoglobin of the blood may vary from zero concentration up to 0.5 per cent of that of ferrous hemoglobin. As we shall see below, a reducing mechanism is present in the red cells to change the ferric hemoglobin back to the ferrous hemoglobin, that is, back to the functional state for oxygen transport. So the level of ferric hemoglobin in the cells is due to the rate at which ferrous hemoglobin is oxidized and the rate at which it is reduced back again.

Ferric hemoglobin is normally produced at a low, constant rate. For example, hemoglobin of normal human hemolyzed erythrocytes maintained in a saline buffer at pH 7.4 at 37°C, in the presence of 95 per cent air and 5 per cent carbon dioxide, was completely converted to ferric hemoglobin in five days.<sup>111</sup> What is surprising is that this rate is so low. We have considered above, the magnetic data which show that the addition of oxygen to ferrous hemoglobin brings about a profound change in electronic configuration with pairing of all the unpaired electrons, and this pairing has been suggested to be connected with the prevention of oxygen from accepting an electron from the ferrous iron. How the ferric hemoglobin arises is not known.

It might be postulated that, for such a transfer of an electron to occur, all that would be necessary would be a momentary loosening of the linkage between the heme iron and the group on the protein to which it is normally attached. This transfer might occur at the moment when an  $O_2$  molecule was being added to ferrous hemoglobin since it has been shown that the rate of methemoglobin formation is proportional to the amount of ferrous hemoglobin rather than to the amount of oxyhemoglobin. Apparently once the  $O_2$  is attached, the bonding of the iron to the globin in oxyhemoglobin is stabilized.

If agents such as amyl nitrite or propiophenone are added to the blood stream a methemoglobinemia is produced. These agents bring about the oxidation of ferrous to ferric hemoglobin. After a time the ferric hemoglobin is reduced back to ferrous hemoglobin. About 0.5 Gm. of ferric hemoglobin in 100 cc. blood per hour are reduced *in vivo* by the enzymes of the normal red cell according to the estimates of Eder, Finch and McKee.<sup>111</sup>

When this reducing mechanism is improperly functioning, too much ferric hemoglobin may accumulate in the cells and result in primary or congenital methemoglobinemia. Sievers and Ryon<sup>112</sup> have demonstrated that the congenital methemoglobinemia is caused by a slow rate of reduction within the red cell rather than by a process which speeds up the rate of oxidation of the hemoglobin as a result of certain drugs. (In general, drugs which bring about oxidation are those which

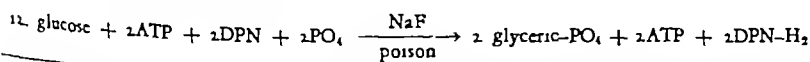
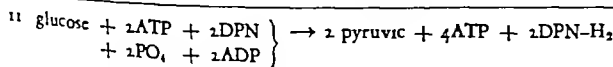
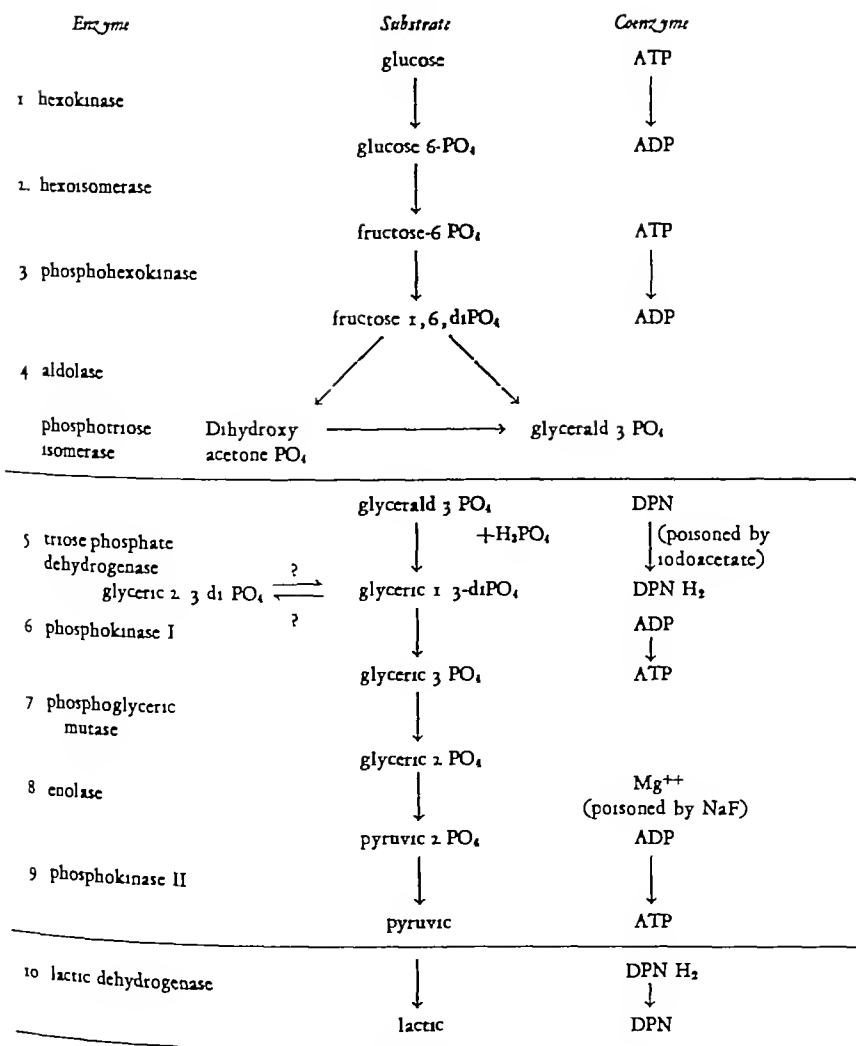


denature the globin, giving rise to hemes which may act as catalysts for oxidation with oxygen <sup>113 114</sup>)

*Glycolytic System of Enzymes and Reduced DPN* In general, the energy released in a mature red cell appears to be derived primarily from the conversion of glucose to lactic acid (fig 5) When glucose is added to red cells lactic acid can be isolated in amounts equivalent to 60-90 per cent of the glucose which has disappeared <sup>11</sup> The glycolysis  $Q_{O_2}^{N_2}$  is + 0.25 and its rate is the same in  $O_2$  as in  $N_2$  The  $O_2$  consumption of the red cells is normally minute, the  $Q_{O_2}$  being -0.05 (i.e., the cu mm  $O_2$  taken up per mg dry wt of red cells per hour) It is not known what material is being oxidized at this slow rate Another enzyme system which has been studied by Dickens (fig 6) appears to oxidize glucose 6-phosphate in a series of oxidative decarboxylations for which the coenzyme required is triphosphopyridine nucleotide (TPN) Parts of this enzyme system have been demonstrated in the red cell This system appears to be of minor importance normally, although in the presence of methylene blue Gibson believes it may play a prominent role in methemoglobin reduction

Two high energy compounds, namely, ATP (adenosine triphosphate) and DPN- $H_2$  (reduced coenzyme) are produced during glycolysis Equation 11 (fig 5) summarizes the process of glycolysis through pyruvate Let us first consider the ATP It is seen from this equation that to bring about the conversion of one glucose molecule to two molecules of pyruvic acid, one must prime the reaction with two high-energy phosphate molecules in the form of ATP The ATP molecules are required for the phosphorylation of steps 1 and 3 (fig 5) However at steps 6 and 9 a total of four high-energy ATP molecules is generated, per glucose molecule broken down by glycolysis The overall gain of high energy phosphate per glucose molecule is thus the conversion of 2 ADP to 2 ATP molecules These high-energy phosphate molecules are in part required for the phosphorylation resynthesis of the pyridine, adenine and riboflavin nucleotides to compensate for the slow hydrolytic breakdown *in vivo* of the coenzymes containing them

The other high-energy compound produced in glycolysis is DPN  $H_2$ , formed by the reduction of DPN at the triose phosphate dehydrogenase stage (step 5) For every glucose molecule glycolyzed through this stage, two molecules of DPN  $H_2$  are formed Since one molecule of DPN- $H_2$  can reduce two hemes of ferric hemoglobin to the functional ferrous hemoglobin, this means that a molecule of glucose according to this scheme would be capable of bring about the reduction of four hemes of ferric hemoglobin It can be calculated that reduction of ferric hemoglobin at the maximum rate of the red cells (0.5 g per 100 cc. blood per hour) would require only about one-tenth the glucose that actually is used up by the red cell If DPN- $H_2$  does not reduce ferric hemoglobin, then at step 10 it will tend to reduce pyruvic to lactic acid However, the reverse reaction of lactic to pyruvic may occur at a reasonable rate only if a relatively high concentration of lactate is present or if a very low concentration of DPN- $H_2$  is present From the redox levels ( $E'_0$  for lactate-pyruvate = -0.180 V,  $E'_0$  for DPN - DPN  $H_2$  = -0.29 V) one may calculate that for a system at equilibrium containing equal concentrations of lactate and pyruvate, the DPN- $H_2$  concentration would only be  $10^{-3}$  that of the



Abbreviations ATP = adenosine triphosphate ADP = adenosine diphosphate DPN = coenzyme I or diphosphopyridine nucleotide or adenine nicotinamide dinucleotide DPN-H<sub>2</sub> = reduced DPN

FIG. 5—STEPS IN THE EMBDEN MEYERHOF GLYCOLYTIC SCHEME (all steps are reversible)

lactate concentration. In other words, if lactate were to be the energy source for the reduction of DPN, the DPN-H<sub>2</sub> would continue to be formed only if it were

rapidly removed from the neighborhood of the enzyme, or if the DPN-H<sub>2</sub> were rapidly oxidized

From these considerations it follows that for the reduction of ferric hemoglobin the production of the reduced coenzyme (DPN-H<sub>2</sub>) from DPN at either the triose phosphate dehydrogenase step or at the lactic dehydrogenase step is essential in the red cell. This has been demonstrated indirectly by Dische,<sup>115</sup> and more directly by Gibson.<sup>116</sup> For example, iodoacetate (0.002 M) is known to poison triose phosphate dehydrogenase and not lactic dehydrogenase. If glucose is used as substrate then in the presence of iodoacetate no reduction of ferric hemoglobin can be found, but addition of lactate can still bring about ferric hemoglobin reduction in the expected ratio of 1 lactate oxidized to 2 ferric hemoglobin reduced. On the other hand, if 0.01 M NaF (which poisons the enolase step by removing Mg<sup>++</sup>, forming the complex MgFPO<sub>4</sub>) is added to the red cells, together with glucose, then reduction can still go on because the triose phosphate dehydrogenase is still active, in this experiment glycemic acid phosphate is found to arise as expected (equation 12, fig. 5). Under normal conditions in the red cell it would appear that reduction of DPN is brought about primarily by the triose phosphate dehydrogenase enzyme.

*Flavine Activity in Methemoglobinemia* In order to reduce ferric hemoglobin to the ferrous state, electrons must pass from DPN-H<sub>2</sub>, the reduced coenzyme, to ferric hemoglobin. This electron transfer does not occur directly to any appreciable extent, and electron mediators appear to be necessary. One important mediator, from analogy with other tissues, is probably a diaphorase enzyme which has flavine adenine dinucleotide as its prosthetic group. It is difficult, however, to conceive that the rate of diffusion of a protein enzyme would be sufficiently rapid to bring about reduction of ferric hemoglobin. Rather does it seem reasonable to postulate that flavine mono- or dinucleotide molecules are present in solution, or that the flavine prosthetic groups are only loosely attached to the protein, i.e., the flavine enzyme is readily dissociated. Thus one may picture the electron as passing from DPN-H<sub>2</sub> → flavine enzyme → flavine nucleotide → ferric hemoglobin.

Determinations of the flavine adenine dinucleotide in normal red cells show it to be in a concentration one-hundredth that of the nicotinamide coenzymes. One suggestion to explain this low content of flavine in normal red cells is that reduced flavine, especially that not attached to proteins, is autooxidizable and can form H<sub>2</sub>O. If much flavine were present, the H<sub>2</sub>O<sub>2</sub> produced might bring about the formation of ferric hemoglobin and the H<sub>2</sub>O<sub>2</sub> would also tend to oxidize the porphyrin ring. It is interesting to note in this connection that in the methemoglobinemias examined by Gibson<sup>116</sup> the metabolic lesion appeared to be caused by a lack of a diaphorase flavine enzyme, rather than by a lack of flavine dinucleotide. This fits in with the findings of Eder et al.<sup>111</sup> who observed no decrease in flavine dinucleotide concentration (as measured by the d-amino oxidase activity) in their methemoglobinemia patients.

*Catalysis of Reduction of Ferric Hemoglobin by Methylene Blue* When as little as  $5 \times 10^{-6}$  M methylene blue is added to red cells in the presence of glucose, the uptake of O<sub>2</sub> is accelerated more than ten fold.<sup>116</sup> Under these conditions, Gibson<sup>116</sup>

found that ferric hemoglobin was reduced at a rate ten times the normal, and with lactate the reduction rate was twice the normal. Glucose brought about the reduction of over 6 moles of ferric heme in the presence of 0.02 M NaF, although the theoretical maximum possible in the glycolytic scheme would be only 4 moles. This suggests that methylene blue is acting as a catalyst for glucose oxidation, which is coupled with reduction of ferric hemoglobin. Gibson postulates that such an oxidation might well take place through the Dickens scheme (fig. 6), where TPN-H<sub>2</sub> could be produced and serve as electron donor.

In general, the action of methylene blue might be explained by its catalyzing the transfer of electrons from reduced coenzymes to ferric hemoglobin. In the glycolytic scheme DPN-H<sub>2</sub> would be involved, and in the Dickens scheme of hexosephosphate oxidation TPN-H<sub>2</sub> would be involved.

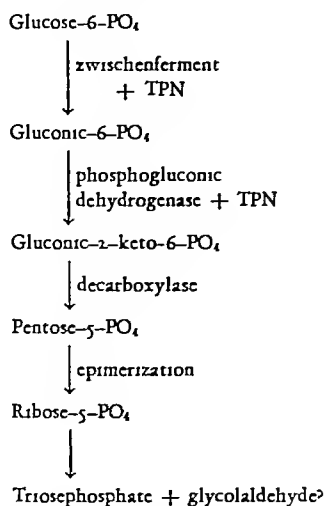


FIG. 6—DICKENS SCHEME<sup>117</sup> FOR THE STEPWISE OXIDATION OF GLUCOSE PHOSPHATE

The enhancement of the reducing ability of normal red cells and also of abnormal red cells of methemoglobinemia cases, when methylene blue was added has led to its use in treatment. Finch<sup>118</sup> has recommended oral administration of 200–300 mg of methylene blue per day to adults with congenital methemoglobinemia, this dose being sufficient to decrease the methemoglobin of the red cells to 1 per cent or less of the total hemoglobin. In this connection it would be interesting to study the effect of feeding riboflavin itself to such patients.

To what extent the tricarboxylic acid cycle is present in the mature erythrocyte cannot as yet be answered. Studies on intact dog red cells by Spicer et al.<sup>128</sup> indicate that citric and succinic acids are ineffective in bringing about reduction of ferric hemoglobin. Whether these substances cannot penetrate the red cell or cannot be metabolized is not known. However, fumaric and malic acids were found to be effective in the reduction of ferric hemoglobin. These results suggest that in the

is present a fumarate hydrating enzyme which converts fumarate to malate and that there is also present a malic dehydrogenase enzyme which oxidizes malate to oxalacetate. This oxidation to oxalacetate is coupled with the reduction of DPN to DPN-H<sub>2</sub>. Nossal<sup>119</sup> has shown that fumarate increases the O<sub>2</sub> uptake of the red cells slightly but more so in the presence of methylene blue. The O<sub>2</sub> uptake in the presence of methylene blue was greater with fructose than with glucose, less with mannose and still less with galactose, ribose and arabinose.

*Reduction of Ferric Hemoglobin by Ascorbic Acid* It has been found in congenital methemoglobinemias that the ascorbic acid of the blood is decreased from a normal value of about 1.5-2.0 mg per 100 cc down to about 0.25 mg/100 cc. The glutathione of the red cells is also found to be decreased from about 40 mg/100 cc blood to 20 mg. Despite adequate diets the ascorbic acid remains low in these cases, suggesting that it might be utilized to some extent in the reduction of ferric hemoglobin.<sup>112</sup>

It has been noted that the maximum ferric hemoglobin arising in congenital methemoglobinemia is between 30-40 per cent of the total hemoglobin. When ferrous hemoglobin solutions are injected intravenously, ferric hemoglobin is formed, likewise if ferric hemoglobin solutions are injected intravenously an equilibrium is established at about 40 per cent ferric hemoglobin. A reducing mechanism must be present which prevents ferric hemoglobin from increasing above this value. It has been suggested that ascorbic acid is the functional reducing agent. Because of the relatively poor reducing ability of ascorbic acid, its functioning becomes appreciable only when a relatively high ferric hemoglobin concentration has developed. Calculations of Sievers and Ryon<sup>112</sup> indicate that the reducing ability of ascorbic acid is far greater than can be accounted for by even stoichiometric reaction. It is possible to explain this action by assuming that ferric hemoglobin in the red cell reacts directly with ascorbic acid and that the dehydroascorbic acid thus formed diffuses back into the blood stream and is reduced to ascorbic acid by other body cells. Satisfactory results in decreasing the methemoglobinemia down to 8-10 per cent methemoglobin have been reported when large doses of ascorbic acid (100-500 mg daily) were fed.

*Glycolysis in Hemolysates* It was observed by Warburg and Christian<sup>120</sup> that after hemolysis of the red cell glucose was no longer utilized and endogenous respiration soon ceased. No reduction of ferric hemoglobin occurred in hemolysates even in the presence of methylene blue. However, if glucose-6-PO<sub>4</sub> were used as substrate then glycolysis could proceed slowly. Evidently some enzyme or coenzyme which was required for the phosphorylation of glucose was destroyed in the hemolysate. Gutman, Jandorf and Bodansky<sup>95</sup> confirmed this work. In addition they found that DPN tended to be hydrolyzed in the hemolysate and that this hydrolysis was diminished by the addition of nicotinamide. They observed considerable reduction of ferric hemoglobin when hexose diphosphate or lactate was used as substrate in the presence of nicotinamide and methylene blue.

The reason for the nonphosphorylation of glucose in hemolysates is suggested by Dische to be due to the absence of ATP.<sup>121</sup> Dische could show that in the presence of 0.02 M NaF or 0.01 M bromoacetate, ATP transphosphorylated with

glucose to form hexosephosphate and triosephosphates so the biochemical lesion in the hemolysate did not lie in the steps above the triosephosphate stage (fig. 5). In a study of the glycolytic enzymes of brain hemogenate, Racker and Krimsky<sup>12</sup> found that triosephosphate dehydrogenase at step 5 (fig. 5) appeared to be the most readily damaged of these enzymes, even being inactivated by traces of iron salts. If the triosephosphate dehydrogenase enzyme were inactivated by hemolysis, it would offer a satisfactory explanation for the nonutilization of glucose since no ATP could then be formed and glucose could not be phosphorylated.

It may be noted in conclusion to this chapter that the metabolic problems of the red cell are not merely of theoretic interest. One of the major practical considerations of wartime research was the development of conditions for the maintenance of the erythrocyte *in vitro* so that whole blood might be shipped to the fighting fronts.<sup>108-123</sup> Under the best conditions, satisfactory preservation of the blood for twenty-one to thirty days was obtained by using an acid-citrate-dextrose\* solution as developed by Loutit & Mollison. The temperature for preservation was 4-10°C. Citrate was used as anticoagulant, glucose was added to maintain glycolysis, and the final acidity of the blood at pH 7.0 seemed to stabilize the enzymes and minimize the ATP changes.

Another practical problem of greater complexity is the maintenance of erythrocytes at 37°C. in the study of malarial infections with the hope that a knowledge of the nutritional requirements of these organisms might lead to a rational chemotherapy.<sup>124-125</sup>

#### SUMMARY

The erythrocyte is a unit of protoplasm highly specialized for the functions of  $O_2$  and  $CO_2$  transport but still containing sufficient repair systems for maintaining itself for about 120 days.

The erythrocyte develops through a complex series of changes, arising from a reticular cell of the bone marrow and differentiating into an actively synthesizing and dividing nucleated cell. After a time the cell stops dividing, the nucleus begins to degenerate and a differentiation takes place in the cytoplasm, the complex mixture of cytoplasmic proteins including mitochondria being replaced almost but not completely by a single kind of protein, namely, hemoglobin.

The functions of several of the anatomic features of the hemoglobin molecule are considered. The hemoglobin molecule has a molecular weight of 68,000 with 4 planar heme units which lie parallel to each other, two being on the proximal and two on the distal surface of the globin. The globin appears to be made up of 4 polypeptide layers with the planar heme units lying perpendicular to the polypeptide layers.

\* Trisodium citrate 2  $H_2O$  = 2.20 g

Citric acid U.S.P. = 0.80 g

Dextrose U.S.P. = 2.45 g

Dilute to 100 cc. with  $H_2O$ . The pH of this solution is about 5.0. Because of its acidity it can be autoclaved without caramelizing the dextrose. This solution is used in the proportion of 15 cc. per 100 cc. blood (generally 75 cc. of fluid per 500 cc. of blood).

The heme units possess a resonating ring structure which stabilizes the unit. The two vinyl groups on the periphery of the heme appear to be necessary if an iron atom is to be inserted into the newly formed protoporphyrin ring. The two ionized propionic acid groups also at the periphery of the heme, appear to be required for orienting and attaching the heme unit to two strongly basic groups of the globin, possibly guanidine groups of arginine. The attachment of the iron of heme is postulated to be to an imidazole nitrogen of a histidine residue of the globin. This latter attachment is by itself a weak bonding but is stabilized not only by the coulombic attraction of the ionized propionic acid groups but also by the Van der Waals forces between the globin and the planar resonating porphyrin.

The attachment of the iron to the imidazole group endows the iron of the heme with the property of combining with  $O_2$  reversibly, the addition of  $O_2$  being connected with a pairing of all the unpaired electrons in the complex. Probably, as a consequence of this pairing of electrons, the  $O_2$  does not act as an oxidant as it would if the special iron link to globin were destroyed. The iron of the heme is bound to 6 atoms or atom groups. It binds 4 nitrogens of the protoporphyrin in the plane of the ring. Below the plane of the ring it binds one nitrogen of the imidazole group, and above the plane of the ring it may then bind  $O$  reversibly. The second nitrogen of the imidazole group is postulated to change its ionization with a change of oxygenation of the hemoglobin, this change in ionization makes possible the conversion of some 50 per cent of the  $CO_2$  transported in the blood to bicarbonate ion, without appreciably changing the pH of the blood. A zinc protein, carbonic anhydrase, is present in the erythrocytes to catalyze the normally slow hydration-dehydration of the  $CO_2-H_2CO_3$  system.

How this non-nucleated erythrocyte is maintained in a functional state for a life span of 120 days is poorly understood. The erythrocyte has a very low  $O_2$  utilization which is compatible with the fact that the mitochondria, which are believed to be the seat of cytochrome oxidase activity, are absent. However, there is present a rather complete glycolytic system which appears to play a major role in the metabolic life of the mature erythrocyte. Hemoglobin is slowly converted in the intact erythrocyte to ferric hemoglobin, i.e., methemoglobin. The methemoglobin is reduced back to the functional ferrous form by reduced diphosphopyridine nucleotide arising during glycolysis. Riboflavin enzymes appear to act as the intermediators between reduced DPN and methemoglobin. The pyridine and flavine enzymes which are slowly undergoing hydrolysis are regenerated by adenosine triphosphate produced in glycolysis. Catalase is present, probably to protect the heme units of hemoglobin against  $H_2O_2$ , the hemes being especially vulnerable to peroxidative attack at the methene links.

#### ACKNOWLEDGMENTS

The author wishes to acknowledge his gratefulness to L. Michaelis, E. Racker and A. Rothen for their suggestions and criticisms.

#### REFERENCES

- <sup>1</sup> MICHAELIS, L. The nature of the interaction of nucleic acids and nuclei with basic dyestuffs. *Cold Spring Harbor Symposia* 12, 131, 1947.
- <sup>2</sup> RHEINGOLD, J., AND WISLOCKI, G. Histochemical methods applied to hematology. *Blood* 7, 641, 1948.

- <sup>1</sup> KAUFMANN, B. P., McDONALD, M., AND GAY, H. Enzymic degradation of ribonucleoproteins of chromosomes, nucleoli and cytoplasm. *Nature* 162 814, 1948
- <sup>2</sup> DEMPSEY, E., AND SINGER, M. Observations on the chemical cytology of the thyroid gland at different functional stages. *Endocrinology* 38 270 1946
- <sup>3</sup> CASPERSSON, T. Ueber den chemischen aufbau der Strukturen des Zellkernes. *Skandinavisches Archiv f. Physiologie* 73 Supplement 8, 1936
- <sup>4</sup> THORELL, B. Studies on the formation of cellular substances during blood cell production. *Acta Med Scandinav Suppl CC* 200 1947
- <sup>5</sup> SABIN, F. R. Bone Marrow. *Cowdry Special Cytology I* ed 2. New York, P. Hoeber, 1932. P. 507
- <sup>6</sup> OSGOOD, E., AND BROWNLEE, I. Culture of human bone marrow. *J A M A* 109 123, 1937
- <sup>7</sup> PLUM, C. Method of marrow culture. *Blood, Special Issue, No. 1* 33, 1947
- <sup>8</sup> PONDER, E. Red cell cytochemistry and architecture. *N Y Acad Sc Annals* 48 579, 1947
- <sup>9</sup> PLUM, C. Studies of erythropoiesis. *Blood Special Issue, No. 1* 42, 1947
- <sup>10</sup> DUSTIN, JR., P. Contribution a l'etude histophysiologique et histochemique des globules rouges des vertebres. *Archives de Biologie* 55 285, 1944
- <sup>11</sup> RIDDLE, K. The endogenous uric acid metabolism in pernicious anemia. *J Clin Investigation* 8 69, 1929
- <sup>12</sup> SHEDDEN, D. Cold Spring Harbor Symposia 1949, in press
- <sup>13</sup> PONDER, E. Hemolysis and Related Phenomena. New York, Grune & Stratton, 1948
- <sup>14</sup> HOGEBOOM, G., SCHNEIDER, W., AND PALADE, G. Cytochemical studies of mammalian tissues. I. Isolation of intact mitochondria from rat liver, some biochemical properties of mitochondria and submicroscopic particulate material. *J Biol Chem* 172 619 1948
- <sup>15</sup> BEACH, E., ERICKSON, B., BERNSTEIN, S., WILLIAMS, H., AND MACY, I. The amino acid composition of erythrocyte posthemolytic residue of five mammalian species. *J Biol Chem* 128 339, 1939
- <sup>16</sup> WILLIAMS, H., ERICKSON, B., AND MACY, I. Chemical structure of the red blood cell. *Quart Rev Biol* 16 80 1941
- <sup>17</sup> PARFART, A. K. Preparation of red cell membrane. *J Cell & Comp Physiol* 19 248, 1942
- <sup>18</sup> HIRST, G. K. Nature of virus receptors of red cells. *J Exp Med* 87 301, 1948
- <sup>19</sup> HORSFALL, F. L., HARDY, P. H., AND DAVENPORT, F. M. Significance of combinations between viruses and host cells. *Bull N Y Acad Med* 24 470 1948
- <sup>20</sup> DE BURON, P. M., PEN-CHUNG YU, HOWE, C., AND BOVARNICK, M. Preparation from human red cells of a substance inhibiting virus hemagglutination. *J Exp Med* 87 1 1948
- <sup>21</sup> HANTO, M. Electrokinetic change in human erythrocytes during adsorption and elution of RR8 influenza virus. *Proc Soc Exper Biol & Med* 68 385 1948
- <sup>22</sup> DAMESHEK, W. Hemolytic mechanisms. *N Y Acad Sc Annals* 48 685 1947
- <sup>23</sup> BOYD, W. C. Fundamentals of Immunology ed 2. New York Interscience Publ., 1947
- <sup>24</sup> SOBENA, A. A., ZINKHAM, W. H., AND WAGLEY, P. F. Observations on the mechanism of hemolysis in paroxysmal cold hemoglobinuria. *Blood* 3 1367, 1948
- <sup>25</sup> SCHMITT, F. O., BEAR, R., AND PONDER, E. The red cell envelope considered as a Wiener mixed body. *J Cell & Comp Physiol* 11 309 1938
- <sup>26</sup> HAUDROWITZ, F., AND STARY, Z. Ueber das Gerüst der Erythrocyten. *Med Klin* 24 740 1928
- <sup>27</sup> WAUGH, D., AND SCHMITT, F. Thickness and ultrastructure of cellular membranes by analytical leptoscope. *Cold Spring Harbor Symposia* 8 233, 1940
- <sup>28</sup> MICHAELIS, L., AND TAKAHASHI, D. Die isoelektrischen Konstanten der Blutkörperchenbestandteile und ihre Beziehungen zur Säurehaemolyse. *Biochem Zeit* 29 439 1910
- <sup>29</sup> HACK, M. H. Estimation of phospholipids in human blood. *J Biol Chem* 169 137 1947
- <sup>30</sup> PARFART, A. Permeability of erythrocyte for anions. *Cold Spring Harbor Symposia* 8 17 1940
- <sup>31</sup> BALLENTINE, R., AND PARFART, A. Action of lipase on the red cell surface. *J Cell & Comp Physiol* 16 49 1940
- <sup>32</sup> JORPES, E. Stroma protein of erythrocytes. *Biochem J* 26 1488 1932
- <sup>33</sup> FURCHGOTT, R., AND PONDER, E. Disk-sphere transformation in mammalian red cells. II. Nature of the anti spherizing factor. *J Exp Biol* 17 127 1940
- <sup>34</sup> CALVIN, M., EVANS, R., BEIERENDT, V., AND CALVIN, G. Rh antigen and haptan antigen and its isolation from erythrocyte stroma. *Proc. Soc Exper Biol & Med* 61 416 1946



- <sup>37</sup> WIENER, A. Blood Groups and Transfusions Springfield Ill. C. C. Thomas 1943
- <sup>38</sup> LANDSTEINER K. Specificity of Serological Reactions Springfield Ill. C. C. Thomas 1936
- <sup>39</sup> KABAT E. BENDICH A. BEYER A. AND BEISER S. Immunochemical studies in blood groups IV Preparation of blood group A substances from human species and a comparison of their chemical and immunochemical properties with those of the blood group A substance from hog stomach J Exp Med 85 685, 1947
- <sup>40</sup> LANDSTEINER K. AND HARTE, R. Group-specific A substances II The substance from hog stomach J Exp Med 71 551 1940
- <sup>41</sup> MORGAN W T J. Occurrence and nature of human blood group substances Brit Med Bull 2 165 1944
- <sup>42</sup> MORGAN W. AND WADDELL M. Specific blood group O substance Brit J Exp Path 26 387 1945
- <sup>43</sup> BENOICH A. KABAT E. A., AND BEYER A. Immunochemical studies on blood groups V Further characterization of blood group A and O substances from individual hog stomachs J Am Chem Soc 69 2163 1947
- <sup>44</sup> SHEMIN D. AND RITTENBERG D. Utilization of glycine for the synthesis of a porphyrin J Biol Chem 159 569 1945
- <sup>45</sup> GRINSTEIN M. AND KAMEN M. unpublished experiments
- <sup>46</sup> ALTMAN K. CASARETT G. MASTERS R. NOONAN T. AND SALOMON K. Hemoglobin synthesis from glycine labeled with radioactive carbon in its alpha carbon atom J Biol Chem 176 319 1948
- <sup>47</sup> GRANICK S. AND GILDER H. Distribution structure and properties of the tetrapyrroles Advances in Enzymology 7 305 1947
- <sup>48</sup> CARTWRIGHT G. E. HUOULEY C. ASCHENBRUCHER H. FAY J. AND WINTROBE M. Studies on free erythrocyte protoporphyrin plasma iron and plasma copper in normal and anemic subjects Blood 3 501 1948
- <sup>49</sup> GROTEFAS W. The porphyrin in normal blood corpuscles Nederland Tijdschr Geneeskunde 81 362, 1937
- <sup>50</sup> LONDON I. M. unpublished observations
- <sup>51</sup> WATSON C. GRINSTEIN M. AND HAWKINSON V. Protoporphyrin IV Comparison of the erythrocyte protoporphyrin concentration with the reticulocyte percentage under experimental and clinical conditions J Clin Investigation 23 69 1944
- <sup>52</sup> GRANICK S. Ferritin its properties and significance for iron metabolism Chem Rev 38 379 1946
- <sup>53</sup> LONDON I. M. SHEMIN D. AND RITTENBERG D. In vitro synthesis of heme in human red blood cell of sickle cell anemia J Biol Chem 173 797 1948
- <sup>54</sup> WYMAN J. Heme proteins Advances in protein chemistry 4. 407 1948
- <sup>55</sup> CARTWRIGHT G. E. Dietary factors concerned in erythropoiesis Blood 2 111 256 1947
- <sup>56</sup> STEIN W., ARCHIBALD R. BRAND, E. CANNAN R. CLARKE, H. EDALL, J. FOSTER G. MOORE S. SHEMIN D. SNELL E. AND VICKERY H. Amino acid analysis of proteins N Y Acad Sci Annals 67 Article 2, 1946
- <sup>57</sup> BRAND E. AND GRANTHAM J. Methionine and isoleucine content of mammalian hemoglobins Its significance for nutritional and metabolic studies J Am Chem Soc 66 724 1946
- <sup>58</sup> BLOCK R. AND BOLLINO, D. Amino Acid Composition of Proteins and Foods Springfield C. C. Thomas 1945
- <sup>59</sup> PORTER R., AND SANGER F. The free amino groups of hemoglobin Biochem J 42 287 1948
- <sup>60</sup> ROOFTON, F. Some recent work on the chemistry of carbon dioxide transport by the blood Harvey Lectures 39 96 1943-44
- <sup>61</sup> WYMAN J. Relation of physiological function and molecular structure in hemoglobin Fed Proceed 7 502, 1948
- <sup>62</sup> PAULING L. Theory of the structure and process of formation of antibodies J Am Chem Soc 62 2643 1940
- <sup>63</sup> BOYES-WATSON J., DAVIDSON E. AND PERUTZ M. An x-ray study of horse methaemoglobin I Proc Roy Soc. A 191 83 1947, A 195 474 1949

- <sup>10</sup> ANSON, M. AND MIRSKY, A. Protein coagulation and its reversal. The preparation of insoluble globin, soluble globin, and heme. *J Gen Physiol* 13 469, 1930
- <sup>11</sup> CORYELL, C. AND PAULINO, L. Structural interpretation of acidity groups associated with the hemes of hemoglobin and hemoglobin derivatives. *J Biol Chem* 132 769, 1940
- <sup>12</sup> PAULINO, L. AND CORYELL, D. Magnetic properties and structure of hemoglobin, oxyhemoglobin and carbon monoxymoglobin. *Proc Natl Acad Sci* 22 210, 1936
- <sup>13</sup> HARTRIDGE, H., AND ROUGHTON, F. The kinetics of haemoglobin. II. The velocity with which oxygen dissociates from its combination with haemoglobin, III. The velocity with which oxygen combines with reduced haemoglobin. *Proc Roy Soc A104* 395 1923 and *A107* 654 1925, resp
- <sup>14</sup> CLARK, W. M. Topics in Physical Chemistry. Baltimore, Williams & Wilkins Co. 1948
- <sup>15</sup> KEILIN, D. AND WANG, Y. Hemoglobin of gastrophilus larvae. Purification and properties. *Biochem J* 40 855, 1947
- <sup>16</sup> ANSON, M., BARCROFT, J., MIRSKY, A. AND OINUMA, S. On the correlation between the spectra of various haemoglobins and their relative affinities for oxygen and carbon monoxide. *Proc Roy Soc. B97* 61 1924
- <sup>17</sup> FOX, H. M. Carbon monoxide affinity of chlorocruorin and haemoglobin. *Nature* 162 20 1948
- <sup>18</sup> HILL, R. AND WOLFEKAMP, H. Oxygen dissociation curve of hemoglobin in dilute solution. *Proc Roy Soc. B120* 484 1936
- <sup>19</sup> GRANICK, S. Some properties of crystalline guinea pig hemoglobin. *J Gen Physiol* 25 571 1942
- <sup>20</sup> BRINKMAN, R., AND JONXIS, J. The occurrence of several kinds of haemoglobin in human blood. *J Physiol* 85 117, 1935
- <sup>21</sup> WITMAN, J. JR., RAFFERTY, J., AND INGALLS, E. Solubility of adult and fetal carbonyl hemoglobin of the cow. *J Biol Chem* 153 275 1944
- <sup>22</sup> VICKERT, H. The histidine content of adult and fetal bovine hemoglobin. *J Biol Chem* 156 283 1944
- <sup>23</sup> SUMNER, J. B., AND SOMERS, G. F. Chemistry and methods of enzymes. N. Y. Acad. Press, 1943
- <sup>24</sup> KEILIN, D., AND HARTREE, E. Properties of catalase. Catalysis of coupled oxidation of alcohols. *Biochem J* 39 293 1945
- <sup>25</sup> SCOTT, D. AND FISHER, A. Carbonic Anhydrase. *J Biol Chem* 144 371 1942
- <sup>26</sup> KEILIN, D., AND MANN, T. Carbonic anhydrase. Purification and nature of the enzyme. *Biochem J* 34 1163 1940
- <sup>27</sup> MANN, T., AND KEILIN, D. Hemocuprein and hepatocuprein. Copper protein compounds of blood and liver in mammals. *Proc Roy Soc. B126* 303 1938
- <sup>28</sup> SCHULTZE, M. The relation of copper to cytochrome oxidase and hematopoietic activity of the bone marrow of rats. *J Biol Chem* 138 219 1941
- <sup>29</sup> PAOET, M. AND VITTO, C. The acid phosphomonoesterase system of human erythrocytes and its sensitivity to the action of sulfanilamide. *Compt rend* 221 594 1945
- <sup>30</sup> ADAMS, D., AND THOMPSON, R. The selective inhibition of cholinesterases. *Biochem J* 42 170 1948
- <sup>31</sup> MENTHA, J., SPRINZ, H. AND BARNARD, R. Rapid extraction of human erythrocyte cholinesterase by alkali pseudoagglutination. *J Biol Chem* 167 623 1947
- <sup>32</sup> STERN, K., REINER, M. AND SILBER, R. Electrophoretic pattern of red blood cell proteins. *J Biol Chem* 161 731, 1945
- <sup>33</sup> PETERS, J., AND VAN SLYKE, D. D. Quantitative Clinical Chemistry. vol. 1. Baltimore, Williams & Wilkins Co. 1946
- <sup>34</sup> POTTER, V. AND FRANK, K. Effect of diet on blood thionine of albino rat. *J Nutrition* 9 1 1935
- <sup>35</sup> CHRISTENSEN, H. AND LYNCH, E. The conjugated nonprotein amino acids of plasma. I. Postabsorptive concentrations of human plasma serum and erythrocytes. *J Biol Chem* 163 741 1946
- <sup>36</sup> DICKE, Z. The breakdown of free and combined adenosine 5 phosphate (AdP<sub>5</sub>) in intact human erythrocytes. *Fed Proc* 7 151 1948
- <sup>37</sup> RACKER, E. Enzymatic formation and breakdown of pentose phosphate. *Fed Proc* 7 180 1948
- <sup>38</sup> LEVITAS, N., ROBINSON, J., ROSEN, F., HUFF, J. AND PERLZWEIG, W. Rapid fluorometric method for determination of total pyridine nucleotides in red blood cells. *J Biol Chem* 167 169 1947

- <sup>92</sup> HANOLER P, AND KOHN, H Cozymase synthesis in the human erythrocyte roles of nicotinic acid and nicotinamide J Biol Chem 150 447, 1943
- <sup>93</sup> LWOFF, A AND LWOFF M Codehydrogenases Proc. Roy Soc London, B122 352, 360 1937
- <sup>94</sup> GUTMANN H JANDORY, B, AND BODANSKY O Role of pyridine nucleotides on reduction of met hemoglobin J Biol Chem 169 145 1947
- <sup>95</sup> MCGOWAN J P Nicotinic acid pentosenucleotides and anemia Nature 144: 244 1939.
- <sup>96</sup> HANDLER P AND FEATHERSTON, W Biochemical defect in nicotinic acid deficiency II Anemia J Biol Chem 151 395 1943
- <sup>97</sup> KLEIN, J R AND KOHN H Synthesis of flavine adenine dinucleotide from riboflavin by human blood cells J Biol Chem 136 177 1940
- <sup>98</sup> CHUIN TONG LINO HROSTED, D AND STARE F The effect of pyridoxine deficiency on the tryptophan niacin transformation in rats J Biol Chem 174 803 1948
- <sup>99</sup> OCHOA, S AND ROSSITER R Flavine adenine dinucleotide in tissues of rats Biochem J 33 2008, 1939
- <sup>100</sup> GIBSON Q H Reduction of methemoglobin by ascorbic acid Biochem J 37 615, 1943
- <sup>101</sup> EISENMANN A, OTT, L SMITH P, AND WINKLER, A Permeability of human erythrocytes to Na and inorganic phosphate by use of radioactive isotopes J Biol Chem 135 165 1940.
- <sup>102</sup> RAPOPORT S, AND GUEST G Change of organic soluble P, diphosphoglycerate, adenosine triphosphate and inorganic P in blood cells of rats during development and healing of rickets J Biol Chem 131 695 1939
- <sup>103</sup> ——— AND ——— Distribution of acid soluble P in the blood cells of various vertebrates J Biol Chem 138 269, 1941
- <sup>104</sup> GUEST, G, AND RAPOPORT S Organic acid soluble P compounds of the blood Physiol Rev 21 49, 1941
- <sup>105</sup> REED, L. AND DENNIS, W Distribution of non protein sulfur of the blood between serum and corpuscles J Biol Chem 73 623 1927
- <sup>106</sup> RAPOPORT, S Dimensional osmotic and chemical changes of erythrocytes in stored blood. J Clin Investigation 26 591, 1947
- <sup>107</sup> GUEST G Methods of preservation of whole blood Advances in Military Med Vol 1 462, 1948. Boston, Little Brown & Co
- <sup>108</sup> ASHBY W Span of life of red blood cell Blood 3 486 1948
- <sup>109</sup> DEAN R NOONAN T, HAEGB, L. AND FENN, W Permeability of erythrocytes to radioactive A. J Gen Physiol 24 353 1941
- <sup>110</sup> EDER, H FINCH C. AND MCKEE R W J Clin Investigation in press
- <sup>111</sup> SIEVERS R AND RYON J Congenital idiopathic methemoglobinemia Arch Inter Med 76 299, 1946
- <sup>112</sup> FINCH, C. Methemoglobinemia and sulfhemoglobinemia New England J Med 239 470 1948
- <sup>113</sup> GRANICK S Iron and porphyrin metabolism in relation to the red blood cell N Y Acad Sc Annals 48 657, 1947
- <sup>114</sup> DISCHE Z Untersuchungen ueber die bedeutung der Phosphorsäureester für den ablauf und die Steuerung der Blutglykolyse I Aufbau der Hexosephosphorsäureester zu Triosephosphorsäureester als erste Stufe der Glykolysebildung eines Fructosephosphorsäureesters aus Glukose und Glyceraldehyd durch intakte Erythrocyten Versuch einer Erklärung der sogenannten Harden youngschen Gärungsgleichung Biochem Zeit 174 51 1934
- <sup>115</sup> GIBSON Q H Reduction of methemoglobin in red blood cells and studies on the course of idiopathic methemoglobinemia Biochem J 42 13 1948
- <sup>116</sup> DICKENS F Oxidation of phosphohexonate and pentose phosphoric acid by yeast enzymes Biochem. J 32 1626 1636 1938
- <sup>117</sup> HAWROP G, AND BARRON E. Effect of methylene blue and other dyes on the oxygen consumption of mammalian and avian erythrocytes J Exp Med 48 207 1928
- <sup>118</sup> NOSSAL, P The metabolism of erythrocytes I Respiration in the absence and presence of methylene blue Anst J Exp Biol & Med Sci 26 223, 1948

- 110 WARBURG O AND CHRISTIAN, W Ueber Aktivierung der Robisonischen Hexose monophosphorsäure in roten Blutzellen und die Gewinnung aktivierender Fermentlösungen *Biochem Z* 242, 205, 1931
- 111 DISCHE A Regulation of phosphorylations in anaerobic glycolysis of red cells by its intermediary products *Fed Proc* 5 131 1946
- 112 RACKER E AND KRIMSKY, I Inhibition of coupled phosphorylation in brain homogenates by ferrous sulfate *J Biol Chem* 173 519 1948
- 113 Series of papers on preservation of blood and red cells in July 1947, issue of *J Clin Investigation*
- 114 Symposium on the biochemistry of malarial parasites *Fed Proc* 5 390, 1946
- 115 TALIAFERRO, W H Biology and biochemistry of the malarial parasites *Advances in Military Med*, Vol 2, 678 1948 Boston Little Brown & Co
- 116 JORDAN H E Evolution of blood forming tissues *Quart Rev Biol* 8 58, 1933
- 117 REDFIELD, A E Evolution of respiratory function of blood *Quart Rev Biol* 8 31, 1933
- 118 SPICER, S S, HANNA, C H, AND CLARK A M Studies in vitro on methemoglobin reduction in dog erythrocytes *J Biol Chem* 177 217, 1949
- 119 WARBURG O Photochemisches Dissoziation des Kohlenoxyd Hemoglobins *Naturforschung und Medizin in Deutschland* 30 (Part 1) 187 1947

# BLOOD CARBONIC ANHYDRASE ACTIVITY IN ANEMIA, WITH A NOTE ON POLYCYTHEMIA VERA

By H D LEWIS, M D , AND M D ALTSCHULE, M D

*With the technical assistance of M TAYLOR*

THE ERYTHROCYTES of the blood contain a number of respiratory enzymes, one of which, carbonic anhydrase, is important in the transport of carbon dioxide. The conversion of carbon dioxide derived from the tissues to bicarbonate in the blood, and the breakdown of bicarbonate to release carbon dioxide in the lungs could not proceed at a rate compatible with health if it were not for the presence of carbonic anhydrase in the red blood cells. This enzyme, widely distributed in nature, is especially abundant in mammalian erythrocytes. Since the enzyme in the blood is contained wholly within the erythrocytes, it is apparent that the carbonic anhydrase activity of the blood might be abnormal in anemia. Although earlier workers have investigated the blood carbonic anhydrase activity in anemic blood,<sup>1-3</sup> their studies were carried out by means of methods which were not strictly quantitative and which had no significance for respiratory function at body temperature. Accordingly it was considered desirable to study this matter again, using a new method.

## MATERIALS AND METHODS

One hundred and twelve observations were made on 85 patients, the latter for the most part were patients with anemias of various types and degrees. Some patients not anemic, were also included in order to control phenomena associated with anemia such as icterus, bone marrow disease, etc. Five of the patients studied had polycythemia vera. The ages and diagnoses are in the tables. All studies were made on venous blood at 37 C by means of a method described elsewhere.<sup>4</sup> The method is a modification of that of Mitchell et al.<sup>5</sup> in that the Warburg apparatus is used; observations are made at 37 C, and calculations of activity are made by extrapolation to undiluted blood from measurements made on three dilutions. In each instance measurements were made in duplicate using three different dilutions of blood so that six measurements were made on each sample. Erythrocyte counts and measurements of hemoglobin content and hematocrit were made on each sample of venous blood used for the estimation of carbonic anhydrase activity. The findings in normal subjects by means of this method<sup>4</sup> are summarized in table 1.

## OBSERVATIONS

### *Pernicious Anemia*

Of the 10 patients studied, 8 were anemic when first seen, the other 2 having responded completely to treatment given previously. All patients studied while anemic showed levels of carbonic anhydrase which were in the normal absolute range, i e., they lay between 1.1 and 2.4 units per ml. of blood (table 2, fig 1).

From the Medical Service and Medical Research Laboratories, Beth Israel Hospital, and the Department of Medicine, Harvard Medical School.

Done in accordance with a contract between the Office of Naval Research, U S Navy, and Harvard University.

In all of the anemic subjects the carbonic anhydrase activity of the blood was high relative to the hemoglobin concentration and erythrocyte counts, in all but 2 the carbonic anhydrase per unit RBC was also above the upper limit of normal, 1 c, 58, at some time (fig. 2). During the course of treatment, absolute values for blood carbonic anhydrase activity rose, however, since hematocrits, hemoglobin levels and erythrocyte counts increased several times, the ratio between carbonic anhydrase activity and these measurements decreased toward normal (table 2, fig. 2). Normal relationships between enzyme activity and hematologic measurements did not regularly obtain, however, until the latter had returned to or almost to normal (table 2, fig. 2).

### *Blood Loss*

Anemia consequent to hemorrhage from peptic ulcer or from carcinoma of the stomach, colon or bladder was associated with a decrease in blood carbonic an-

TABLE 1—Observations in Forty-two Normal Subjects

	Range	Average
Hematocrit (per cent erythrocytes)	38.0–56.0	45.2
Hemoglobin (grams per ml blood)	0.133–0.199	0.138
Erythrocytes (billions per ml blood)	3.82–5.93	4.87
Carbonic Anhydrase (units per ml blood)	1.2–2.6	1.8
Carbonic Anhydrase (units per ml erythrocytes)	2.6–5.8	4.05
Carbonic Anhydrase (units per gram of hemoglobin)	8–17	13
Carbonic Anhydrase (units per billion erythrocytes)	0.25–0.56	0.37

hydrase activity (table 3, fig. 3). The fall in activity of the enzyme paralleled decreases in hematocrits, hemoglobin levels and erythrocyte counts, so that the ratio of carbonic anhydrase activity to these measurements was in the normal range in every instance (table 3, fig. 4). Neither the cause of the bleeding nor its chronicity influenced the level of activity of the blood carbonic anhydrase, the 3 patients (Cases 15, 18 and 20, table 3) in whom the anemia had been present for months with a resultant decrease in cell size, showed relationships between enzyme activity and the various other measurements made on the blood which were similar to those found in patients with bleeding of recent onset, changes in cell size had no significant effect.

### *Infection*

Slight anemia was encountered in 10 patients in association with chronic febrile diseases, these included rheumatoid arthritis, rheumatic fever, pyelonephritis, pulmonary tuberculosis and ulcerative colitis. The carbonic anhydrase activity was slightly lowered but in every case lay within the range of normal in keeping with the mildness of the anemia (table 3, figs. 4 and 5), the presence of persistent diarrhea (Cases 30 and 31, Table 3) did not influence the findings.

*Uremia*

The anemia of uremia likewise was found to be accompanied by a decrease in blood carbonic anhydrase level, the diminution in activity of the enzyme in the blood paralleled the severity of the anemia so that the ratio between carbonic

TABLE 2.—*Observations in Patients with Pernicious Anemia*

Case	Age	Sex	Date	Hematocrit per cent erythrocytes	Carbonic Anhydrase units per ml. blood	Carbonic Anhydrase units per ml. erythrocytes
1	75	M	4 18-47	17.5	1.3	7.4
			4 30	30.3	1.8	5.9
			5-6	32.3	2.1	6.5
			5 15	34.1	2.3	6.8
2	70	F	4 24 47	27.2	1.6	5.9
			5 8	37.0	1.8	4.9
			5 28	35.1	1.6	4.6
3	59	F	5 21 47	41.8	2.3	5.5
4	66	M	8-5 47	15.0	1.9	12.7
			8 19	26.2	1.9	7.2
			8 27	31.5	2.4	7.6
			3 2 48	44.8	1.5	
			4 20-48	43.0	1.5	
5	56	F	11 17 47	43.0	1.4	3.3
6	40	F	12 16-47	22.0	1.1	5.0
			1 13 48	41.0	2.2	5.3
7	81	F	1 21 48	14.3	1.8	12.7
			1 27	14.7	1.8	12.1
			2-11	26.5	2.2	8.3
			4 16	41.0	1.3	
8	85	F	1 28 48	15.6	1.4	9.0
9	64	F	2-11 48	23.3	1.3	5.7
			2 18	26.5	1.4	5.3
10	82	M	7 7 48	21.0	2.2	10.6

anhydrase activity and the hematocrits, hemoglobin levels and erythrocyte counts remained normal (table 3, figs 4 and 5) The severity of acidosis did not influence the findings

*Hepatic Disease*

Of the eight patients with cirrhosis studied, four were anemic and the others not. In all of them but one the ratios of carbonic anhydrase activity to hematocrits,

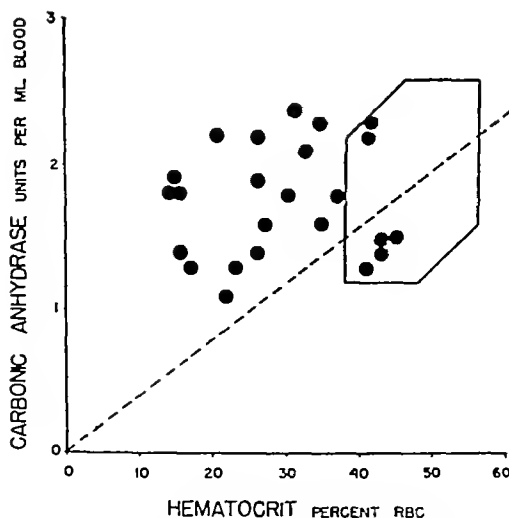


FIG 1.—PERNICIOUS ANEMIA RELATION BETWEEN CARBONIC ANHYDRASE ACTIVITY OF WHOLE BLOOD AND HEMATOCRIT. The parallelogram indicates the normal range the dotted line is drawn from the origin through the average normal value.

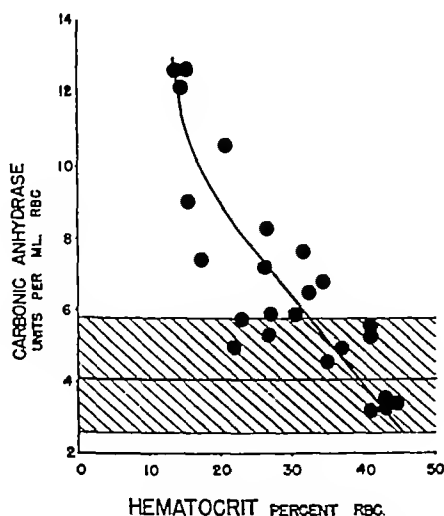


FIG 2.—PERNICIOUS ANEMIA RELATION BETWEEN CARBONIC ANHYDRASE ACTIVITY OF ERYTHROCYTES AND HEMATOCRIT. The cross hatched area is the normal range for carbonic anhydrase activity per ml erythrocytes the heavy line through it is the level of the normal mean value.

hemoglobin levels and erythrocyte counts were normal (table 3, fig 6), the one exception (table 3, Case 42) had an excessive amount of the enzyme for the degree of anemia he presented and one of those with normal ratios (Case 37) was at the



TABLE 3—Observations in Patients with Various Conditions

Case	Age	Sex	Hematocrit per cent erythrocytes	Carbonic Anhydrase units per ml blood	Carbonic Anhydrase units per ml erythrocytes	
11	52	M	19 0 28 0	0 88 1 1	4 6 3 9	Hemorrhage 1 week later
12	66	M	11 8	0 46	3 9	Hemorrhage
13	72	M	38 0	1 7	4 5	Hemorrhage
14	26	F	33 0 36 5	1 0 1 6	3 0 4 4	Hemorrhage 2 weeks later
15	67	M	36 2	1 5	4 1	Hemorrhage
16	89	F	30 0	1 2	4 0	Hemorrhage
17	53	M	15 1	0 7	4 7	Hemorrhage
18	43	M	29 5	1 5	5 1	Hemorrhage
19	54	F	41 0	1 5	3 8	Hemorrhage
20	78	M	39 5	1 3	3 3	Hemorrhage
21	75	M	25 5	1 3	5 1	Hemorrhage
22	56	F	26 5	1 2	4 5	Hemorrhage
23	28	F	38 0	1 4	3 7	Chronic Infection
24	32	M	37 0	1 9	5 1	Chronic Infection
25	57	M	36 9	1 3	3 5	Chronic Infection
26	69	F	42 0	1 3	3 1	Chronic Infection
27	45	F	39 2	1 3	3 3	Chronic Infection
28	62	F	39 0	1 8	4 6	Chronic Infection
29	70	F	35 0	1 4	4 0	Chronic Infection
30	47	F	32 5	1 6	4 9	Chronic Infection
31	35	F	32 0	1 2	3 7	Chronic Infection
32	31	F	38 0	1 4	3 7	Chronic Infection
33	55	M	23 0	1 0	4 3	Uremia

TABLE 3—Continued

Case	Age	Sex	Hematocrit per cent erythrocytes	Carbonic Anhydrase units per ml blood	Carbonic Anhydrase units per ml erythrocytes	
34	65	M	39 0 20 5	1 3 0 81	3 2 3 9	Uremia 5 weeks later
35	72	M	32 0	1 4	4 4	Uremia
36	50	M	34 0	1 2	3 5	Uremia
37	36	F	36 0	2 0	5 6	Portal Cirrhosis
38	45	F	44 7	1 3	2 9	Portal Cirrhosis
39	68	M	40 5	1 3	3 2	Portal Cirrhosis
40	55	M	38 5	1 5	4 0	Portal Cirrhosis
41	38	M	29 5	1 3	4 4	Portal Cirrhosis
42	66	M	33 5 42 8	2 0 1 6	6 0 3 7	Portal Cirrhosis, hemorrhage After 1500 blood I V
43	49	M	43 5	1 4	3 2	Biliary cirrhosis, severe icterus
44	52	M	51 0	1 7	3 3	Hemachromatosis
45	67	M	40 3	1 3	3 5	Cancer of pancreas, severe icterus
46	78	F	34 0	1 4	4 1	Cancer of liver severe icterus
47	68	M	20 4 24 2 23 0 27 0	0 70 1 0 1 1 2 0	3 4 4 1 4 8 7 4	Chronic myelogenous leukemia After 3 weeks After 6 weeks After 7 months
48	54	F	28 2	1 0	3 5	Chronic myelogenous leukemia.
49	49	F	26 7 35 4 31 8	1 2 1 7 1 7	4 5 4 8 5 3	Chronic myelogenous leukemia After 1 week. After 3 weeks
50	45	M	29 9	1 8	6 0	Chronic myelogenous leukemia.
51	48	F	35 1	2 3	6 5	Chronic myelogenous leukemia.
52	33	F	35 0	2 1	6 0	Chronic myelogenous leukemia
53	47	M	31 0	1 5	4 8	Chronic myelogenous leukemia.
54	47	F	29 4 35 5	1 6 1 7	5 3 4 8	Chronic myelogenous leukemia. After 3 months

TABLE 3—Continued

Case	Age	Sex	Hematocrit per cent erythrocytes	Carbonic Anhydrase units per ml blood	Carbonic Anhydrase units per ml erythrocytes	
55	37	M	51.3	1.5	2.9	Acute myelogenous leukemia.
56	62	M	21.2 27.0	0.86 1.1	4.1 4.1	Chronic lymphatic leukemia. After 3 months
57	61	M	35.0	1.1	3.1	Chronic lymphatic leukemia.
58	40	F	37.4	1.6	4.3	Lymphoma.
59	56	M	40.0	1.5	3.7	Lymphoma.
60	19	M	36.0	1.3	3.6	Lymphoma.
61	68	F	35.5	1.4	3.9	Hodgkin's Disease.
62	63	F	33.7	1.5	4.5	Multiple Myeloma.
63	54	F	26.2	0.9	3.4	Plasma cell leukemia.
64	57	F	30.0 30.5	1.8 1.9	6.0 6.2	Refractory anemia After 3 months.
65	56	F	36.7	2.0	5.4	Refractory anemia.
66	55	F	27.2	2.1	7.7	Refractory anemia.
67	48	M	37.5	1.5	4.0	Refractory anemia.
68	63	M	17.9	1.2	6.4	Multiple deficiencies.
69	46	F	39.5	1.8	4.6	Scurvy
70	58	F	36.6	1.5	4.1	Aplastic anemia
71	54	F	31.6	1.1	3.5	Aplastic anemia
72	22	F	28.0 34.0	1.8 1.5	6.4 4.4	Sickle cell anemia After 1 month
73	20	M	30.2	1.4	4.6	Sickle cell anemia
74	24	F	35.0	1.7	4.9	Infectious mononucleosis.
75	30	F	36.0	1.7	4.7	Cooley's anemia.
76	55	M	22.0	1.1	5.0	Acute Hemolytic Anemia
77	43	M	25.1	1.2	4.6	Paroxysmal Nocturnal Hemoglobinuria

TABLE 3—*Concluded*

Case	Age	Sex	Hematocrit per cent erythrocytes	Carbonic Anhydrase units per ml blood	Carbonic Anhydrase units per ml erythrocytes	
78	32	F	39 0	1 6	4 1	Familial Hemolytic Icterus
79	34	F	36 0	1 7	4 7	Anorexia nervosa
80	28	F	33 0	2 0	6 1	Diabetes mellitus malnutrition
81	56	M	69 2	2 7	3 9	Polycythemia Vera
81	60	M	69 2	3 1	4 5	Polycythemia Vera
83	80	M	56 5	1 8	3 2	Polycythemia Vera
84	55	F	60 0	2 1	3 5	Polycythemia Vera
85	55	F	67 5	2 6	3 9	Polycythemia Vera

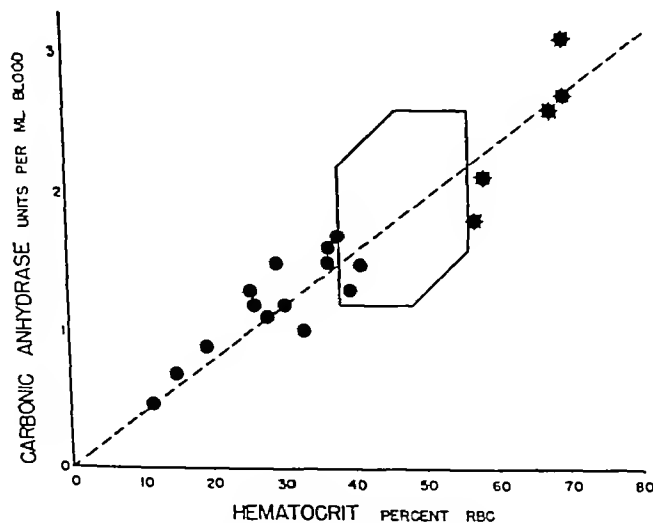


FIG 3—ANEMIA OF BLOOD LOSS POLYCYTHEMIA VERA RELATION BETWEEN CARBONIC ANHYDRASE ACTIVITY OF WHOLE BLOOD AND HEMATOCRIT The crellated dots indicate polycythemia vera The parallelogram indicates the normal range the dotted line is drawn from the origin through the average normal value

upper range of normal The presence of icterus caused no deviation from the carbonic anhydrase activity expected on the basis of the hematological findings

#### *Leukemia and Allied Conditions*

Anemia was present in all of the 17 patients studied, with the exception of one, who had acute myelogenous leukemia (table 3, Case 53) When anemia was present,

the blood carbonic anhydrase activity was reduced as a rule to or below the lower range of normal, the ratio between enzyme activity and hematocrits, hemoglobin levels and erythrocyte counts remaining normal (table 3, fig 7) In 4 patients, however, (table 3, Cases 45, 48, 49, 50) who comprised half of the patients with

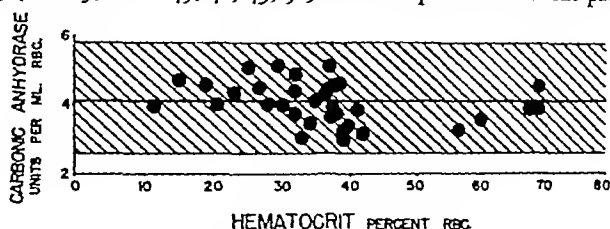


FIG 4.—ANEMIAS OF BLOOD LOSS, INFECTION AND UREMIA. POLYCYTHEMIA VERA. RELATION BETWEEN CARBONIC ANHYDRASE ACTIVITY OF ERYTHROCYTES AND HEMATOCRIT. The cross-hatched area is the normal range for carbonic anhydrase activity per ml erythrocytes, the heavy line through it is the level of the normal mean value.

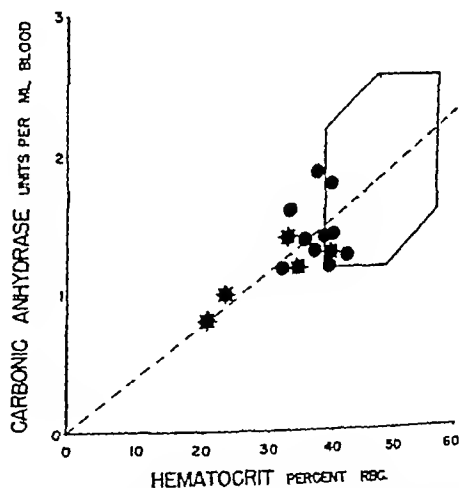


FIG 5.—ANEMIAS OF INFECTION AND OF UREMIA. RELATION BETWEEN CARBONIC ANHYDRASE ACTIVITY OF WHOLE BLOOD AND HEMATOCRIT. The circled dots indicate uremia. The parallelogram indicates the normal range, the dotted line is drawn from the origin through the average normal value.

chronic myelogenous leukemia, the blood carbonic anhydrase level was high in the normal range in spite of the presence of anemia, so that the ratio between enzyme activity and hematocrits, hemoglobin levels and erythrocyte counts was abnormally high.

#### *Miscellaneous Anemias*

Studies on instances of various uncommon types of anemia revealed, with a few exceptions, blood carbonic anhydrase levels in or below the lower normal range.

decreases in enzyme activity paralleled the severity of anemia so that the ratio between carbonic anhydrase level and the hematocrits, hemoglobin levels and

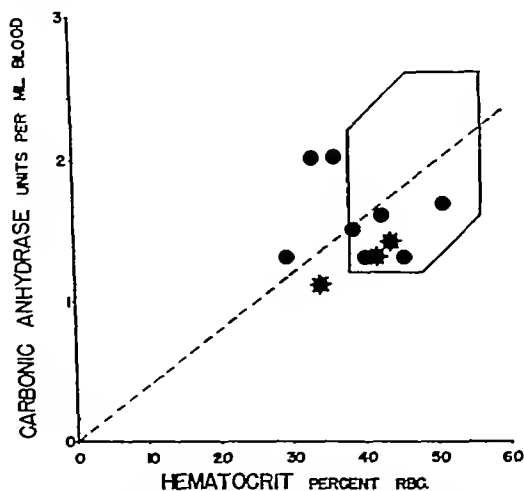


FIG 6—HEPATIC DISEASE RELATION BETWEEN CARBONIC ANHYDRASE ACTIVITY OF WHOLE BLOOD AND HEMATOCRIT The crellated dots indicate jaundice, the parallelogram indicates the normal range, the dotted line is drawn from the origin through the average normal value

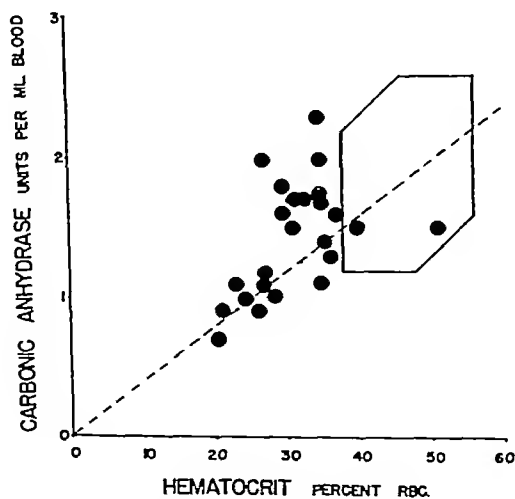


FIG 7—LEUKEMIA RELATION BETWEEN CARBONIC ANHYDRASE ACTIVITY OF WHOLE BLOOD AND HEMATOCRIT The parallelogram indicates the normal range the dotted line is drawn from the origin through the average normal value

erythrocyte counts were normal. Exceptions to this finding were encountered in 2 patients with dietary malnutrition (table 3, Case 77) and 2 of the 4 instances of

refractory anemia (table 3, Cases 62 and 64), the 2 other cases of refractory anemia (table 3, Cases 63 and 65) revealed ratios in the normal range

### *Polycythemia Vera*

Patients with polycythemia vera showed values for blood carbonic anhydrase activity high in or above the normal range, depending on the severity of the condition (table 3, fig 3) The ratios between enzyme activity and the hematocrits, hemoglobin levels and erythrocyte counts were normal (fig 4)

### DISCUSSION

The present study for the most part is in qualitative agreement with the earlier observations of Lambie<sup>2</sup> on anemia. Different types of anemia vary in regard to the relation between red cell mass and carbonic anhydrase activity of the blood. In the commonly encountered anemias consequent to loss of blood, infection and uremia and probably also in the less common aplastic and hemolytic anemias, a decrease in erythrocytes signifies not only a parallel loss of hemoglobin, but also a corresponding diminution in carbonic anhydrase activity of the blood. The same condition also obtains in most patients with anemia associated with hepatic disease and with leukemia and allied conditions. On the other hand, in patients with pernicious anemia and in some instances of refractory anemia, of hepatic disease and of myelogenous leukemia, the blood carbonic anhydrase activity remains in or only slightly below the normal range in spite of marked decreases in hematocrit, hemoglobin level and erythrocyte count. Patients with pernicious anemia have extremely high blood carbonic anhydrase activity relative to erythrocyte count and exhibit levels of enzyme activity which may be several times as high as that shown by patients with comparable hemoglobin or hematocrit levels associated with anemia of blood loss, infection or uremia. The reason for this difference is not known. In contradistinction to earlier workers<sup>1-3</sup> no evidence was found to support the concept that icterus increases blood carbonic anhydrase activity.

The precise significance of the findings of the present study cannot be stated in the absence of complete information as to the physiological function of the blood carbonic anhydrase. Theoretical considerations indicate that its property of accelerating the reaction  $\text{H}_2\text{O} + \text{CO}_2 \rightleftharpoons \text{HCO}_3^-$  is essential for the prevention of accumulation of carbon dioxide in the body. It appears, therefore, that under the conditions of accelerated blood flow through the tissues and lungs which obtain in anemia,<sup>6</sup> the need for carbonic anhydrase is greater than normal.

There is no experimental evidence available at present which proves that loss of blood carbonic anhydrase activity definitely causes dyspnea. Sulfanilamide inhibits carbonic anhydrase and therefore observations on sulfanilamide intoxication are pertinent to the problem of dyspnea. The clinical observation that the administration of sulfanilamide causes increased respiratory activity,<sup>7-9</sup> and intolerance to exercise<sup>10</sup> and to inhalation of carbon dioxide,<sup>11</sup> is difficult to interpret, in clinical conditions of sulfonamide intoxication not only is the activity of the carbonic anhydrase of the blood depressed, but that of the renal tubular carbonic anhydrase probably is also, with the consequent development of acidosis.

due to loss of base. Changes in blood and urinary chemistry over a period of time after administration of sulfanilamide are so complicated as to suggest the effects of the action of several factors.<sup>7-9</sup> The work of Wood and Favour<sup>9</sup> showed, however, that the injection of sulfanilamide intravenously rapidly causes inhibition of the enzyme in the blood and that immediately thereafter lowering of the arterial blood carbon dioxide content occurs. This observation suggests that decreased carbonic anhydrase activity in the blood may cause or contribute to dyspnea through impaired removal of carbon dioxide from the tissues, accumulation of carbon dioxide in the brain causes stimulation of respiration with consequent hyperventilation and immediate lowering of arterial blood carbon dioxide tension. Apparently accumulation of carbon dioxide in the blood through retardation of its excretion in the lungs is not a factor. The fall in arterial blood carbon dioxide level which occurs as a consequence of inhibition of carbonic anhydrase activity by administration of sulfanilamide resembles the decrease in blood carbon dioxide usually found in patients with anemia<sup>6</sup>, however, in these patients additional factors, such as anoxia and also impaired heat dispersal consequent to cutaneous vasoconstriction, also cause hyperventilation. Data now available do not permit distinction between hyperventilation possibly due to lack of carbonic anhydrase and that consequent to other factors in patients with anemia.

The numerous and complex cardiovascular and respiratory compensations in anemia have been discussed elsewhere.<sup>6</sup> The importance of erythrocytes in carbon dioxide transport is established. Although the red blood cells hold less carbon dioxide than plasma, they take up approximately 40 per cent of the carbon dioxide added to the blood as it circulates through the tissues. The mechanisms whereby erythrocytes are able to hold so much carbon dioxide at a pH of 7.1 in competition with plasma whose pH is 7.4 have not been delineated completely. Several factors have been studied. Hemoglobin is a buffer, change in its acidity when it is reduced accounts for an important part of the carbon dioxide carrying power of erythrocytes, carbonic anhydrase is important in this phenomenon, for without the enzyme bicarbonate cannot enter the red cells in a normal fashion.<sup>12-13</sup> Similarly the chloride shift cannot occur at a normal speed in the absence of adequate amounts of active carbonic anhydrase in erythrocytes.<sup>13-15</sup> Another possible factor, not completely studied, is the transport of carbon dioxide in the form of carbamates in combination with hemoglobin and possibly other substances, it is probable that still other factors also operate. Whether deficiency of blood carbonic anhydrase interferes with carbon dioxide transport solely through impairment of mechanisms involving hemoglobin or whether other factors also play a part is not known, the lack of complete knowledge as to the precise function of carbonic anhydrase in blood gas transport makes it impossible at the present time to examine critically the role of the enzyme in the production or prevention of symptoms.

Although hemoglobin is essential for oxygen transport and very important in carbon dioxide transport, the fact remains that patients with pernicious anemia have long been known to tolerate exertion without the development of severe dyspnea even when the blood hemoglobin level is as low as that which is associated with dyspnea in patients with some other chronic anemias, such as those consequent to slow loss of blood. This fact indicates the importance of factors



other than hemoglobin level in the genesis of the dyspnea of anemia and suggests that the observed differences in blood carbonic anhydrase activity might be significant in this regard

In polycythemia vera, the increases in blood carbonic anhydrase activity which parallel increases in hematocrit apparently have no vital importance in the altered cardiorespiratory function which occurs in this disease

### SUMMARY AND CONCLUSIONS

Measurements of blood carbonic anhydrase activity were made in patients with a variety of blood dyscrasias, using a new method. In patients with anemia due to loss of blood, infection and uremia, and in most of those with anemia associated with liver disease and leukemia, a constant relation was found between blood carbonic anhydrase activity and the hematocrit, the same holds in polycythemia vera. In patients with pernicious anemia, and in some with refractory anemia, and anemias associated with hepatic disease and with myelogenous leukemia, blood carbonic anhydrase activity was in or near the normal range in spite of lowered hematocrit values. The possible relation between these differences among anemias and the tolerance of patients with various anemias to exercise is discussed.

### REFERENCES

- <sup>1</sup> HODGSON, T. H. The carbonic anhydrase content of blood in pathological states in man. *Brit. J. Exp. Path.* 27: 75, 1936.
- <sup>2</sup> LAMBIE, C. G. The functional pathology of anemia. Part II. The transport and elimination of carbon dioxide. *M. J. Australia* 2: 341, 1937. The functional pathology of anemia. Part III. Restoration of compensation tolerance and failure. *Med. J. Australia* 2: 378, 1937.
- <sup>3</sup> ———. Observations on the carbonic anhydrase of the blood in anemias and in other pathological conditions. *Edinburgh M. J.* 45: 373, 1938.
- <sup>4</sup> ALTSCHULE, M. D. AND LEWIS, H. D. Measurement of carbonic anhydrase activity of blood at body temperatures. *J. Biol. Chem.* in press.
- <sup>5</sup> MITCHELL, C. A., POZZANI, V. C. AND FERRENDEN, R. W. A method for determining carbonic anhydrase activity by the use of unimolecular velocity constants. *J. Biol. Chem.* 166: 283, 1945.
- <sup>6</sup> BLUMHART, H. L., AND ALTSCHULE, M. D. Clinical significance of cardiac and respiratory adjustments in chronic anemia. *Blood* 3: 329, 1948.
- <sup>7</sup> HARTMANN, A. F., PERLEY, A. M. AND BARNETT, H. L. A study of some of the physiological effects of sulfanilamide. I. Changes in the acid base balance. *J. Clin. Investigation* 17: 645, 1938.
- <sup>8</sup> BECKMAN, W. W., ROSENBLUM, E. C., PETTENHILL, R. B. AND BAUER, W. A study of the effects of sulfanilamide on acid base metabolism. *J. Clin. Investigation* 19: 635, 1940.
- <sup>9</sup> WOOD, W. B. JR., AND FAVOUR, C. B. The relation of sulfanilamide acidosis to the specific inhibition of carbonic anhydrase. *J. Clin. Investigation* 20: 433, 1941.
- <sup>10</sup> ROUGHTON, F. J. W., DILL, D. B., DARLING, R. C., GRAYBIE, A., KNEER, C. A. AND TALBOTT, J. H. Some effects of sulfanilamide on man at rest and during exercise. *Am. J. Physiol.* 135: 77, 1941.
- <sup>11</sup> LOCKE, A., MAIN, E. R. AND MELLON, R. R. Carbonic anhydrase inactivation as the source of sulfanilamide acidosis. *Science* 93: 66, 1941.
- <sup>12</sup> JACOBS, M. H. AND STEWART, D. R. The role of carbonic anhydrase in certain ionic exchanges involving the erythrocyte. *J. Gen. Physiol.* 25: 339, 1941.
- <sup>13</sup> KELLIN, D., AND MANN, T. Activity of carbonic anhydrase within red blood corpuscles. *Nature* 161: 493, 1941.
- <sup>14</sup> BOOTH, V. H. Carbonic anhydrase activity inside corpuscles. *Enzyme—substrate accessibility factors.* *J. Physiol.* 93: 117, 1938.
- <sup>15</sup> FEOLER, J. Function of carbonic anhydrase in blood. *Nature* 153: 137, 1944.

# THE ZINC CONTENT OF WHOLE BLOOD, PLASMA, LEUKOCYTES AND ERYTHROCYTES IN THE ANEMIAS

By BERT L. VALLEE, M.D., AND JOHN G. GIBSON, II, M.D.

WE HAVE previously described our findings of the zinc content of normal whole blood, plasma, leukocytes and erythrocytes.<sup>1</sup> The following zinc concentrations in micrograms (gammas) were found to represent the normal pattern

	<i>Amis</i> in $\mu\text{g}$	<i>S.D.</i>	<i>Unit</i>
Whole Blood	88	$\pm 2.0$	1 cc.
Plasma	30	$\pm 1.6$	1 cc.
Leukocytes	$3.2 \times 10^{-2}$	$\pm 1.3 \times 10^{-2}$	1 million cells
Erythrocytes	$1.34 \times 10^{-2}$	$\pm 0.2 \times 10^{-2}$	1 million cells
Erythrocytes	144	$\pm 2.7$	1 cc.*

Sutton and Nelson,<sup>2,3</sup> and Smith and Larson<sup>4</sup> fed zinc to rats and observed the effect on erythropoiesis and leukopoiesis as reflected in the cytology of the peripheral blood. No measurements of the zinc content of red and white cells were carried out, however. No quantitative measurements of the zinc concentrations of the blood components in patients with anemia are on record. The study herewith reported was conducted on patients afflicted with various types of anemia, in an attempt to elucidate the role of zinc in erythrocytes.

## METHOD

The hematologic techniques and method of measuring blood zinc content have been described previously.<sup>1,5,6</sup> The small number of patients which was studied in each category precluded internal statistical analysis. The mean of the normal series was taken as a point of reference in the evaluation of individual values. Zinc concentrations lying outside of two standard deviations were considered abnormal. For two standard deviations one would expect 1 out of 20 observations on normals to fall outside of these limits.

## MATERIAL STUDIED

Nine patients with pernicious anemia were studied. Samples were obtained prior to or within a few days following institution of liver therapy. Five of these patients were followed over a prolonged period of time while under maintenance therapy with liver extract. A total of 34 blood samples was analyzed.

A second group of patients with various types of anemia was investigated. Five patients were found to have so-called refractory anemia. Three of these patients had white blood cell counts below 2000/mm.<sup>3</sup> Four patients were anemic secondary to hemorrhage: 2 had an anemia of infection, 2 had a sickle cell anemia. 1 patient each had nutritional anemia, iron deficiency anemia, splenic anemia, infectious mononucleosis, and Cooley's anemia. There was a total of 23 blood samples.

## RESULTS

Table 1 summarizes the zinc concentrations found in whole blood, plasma, leukocytes and erythrocytes in patients with anemias other than pernicious anemia.

From the Department of Medicine, Harvard Medical School and the Medical Clinic of the Peter Bent Brigham Hospital, Boston, and the Department of Physics, Massachusetts Institute of Technology, Cambridge, Mass.

This study was supported by a grant in aid from the National Institute of Health.  
\* 1 cc. of erythrocytes packed by centrifuging at 3000 r.p.m. for 30 minutes.

mia Table 2 presents similar data obtained from patients with pernicious anemia. The values for the zinc concentrations contained in 1 ml of whole blood due to plasma, leukocytes and erythrocytes are shown in tables 3 and 4. These values were calculated as previously described.<sup>1</sup>

The abnormal red blood cell findings are essentially limited to patients with pernicious anemia and possibly sickle cell anemia. In pernicious anemia, in contrast to other forms of anemia, in which zinc concentrations were found to be in the

TABLE 1—The Unit Values of Zinc Content of Whole Blood, Plasma, Leukocytes and Erythrocytes in Anemias other than Pernicious Anemia

Exp No	Diagnosis	RBC per mm. <sup>3</sup>	Hct %	M C V in $\mu^3$	Unit Content by Dithizone Extraction				
					Whole Blood	Plasma	Leuko- cytes	Erythrocytes	
					$\gamma$ per ml		$\gamma \times 10^{-1}$ per $1 \times 10^6$ cells	$\gamma \times 10^{-1}$ per $1 \times 10^6$ cells	Zn $\gamma$ /cc, packed red cells Corr for M.C.V.
142	Refractory Anemia	324	24.2	74.5	13.5	5.5	3.3	1.49	17.7
171	Refractory Anemia	365	20.0	55.0	3.0	1.5	16.1	—*	—*
172	Refractory Anemia	360	31.6	88.0	5.6	2.1	55.5	0.92	10.0
173	Refractory Anemia	342	29.9	87.5	4.2	1.3	16.8	1.09	9.5
181	Refractory Anemia	366	36.6	100.0	11.5	2.8	19.0	1.60	16.4
621	Refractory Anemia	373	36.7	90.5	8.1	4.4	29.0	1.33	13.6
95-1	Refractory Anemia	322	30.0	93.2	8.2	3.7	1.8	2.06	22.3
811	Hemorrhage	268	24.0	90.0	5.7	1.3	1.1	1.69	18.9
106-1	Hemorrhage	352	36.2	88.6	12.8	9.1	2.6	1.92	18.8
1181	Hemorrhage	292	24.0	82.2	7.1	—5	3.9	1.26	15.4
1531	Hemorrhage	293	25.5	87.0	6.8	3.0	1.4	1.45	16.8
49-1	Nutritional	332	33.0	90.5	17.8	21.7	4.1	1.62	16.4
139-1	Iron Deficiency	476	37.5	79.0	6.7	1.2	—*	1.01	12.8
39-1	Uremia	325	30.6	94.5	7.0	2.7	4.0	1.43	15.2
1121	Uremia	295	28.7	97.0	5.9	2.6	4.4	1.65	16.7
981	Sickle Cell	395	31.0	78.0	12.5	4.2	1.6	2.01	25.8
982	Sickle Cell	385	34.0	88.0	7.2	1.4	—*	1.50	17.0
1331	Sickle Cell	329	32.0	97.5	16.3	4.2	1.6	1.91	19.6
56-1	Splenic	399	31.6	79.0	8.4	4.6	6.4	1.51	19.3
66-1	Infection	396	39.0	99.0	11.2	4.5	1.6	1.93	19.8
1071	Infection	378	36.9	97.5	10.4	4.3	6.6	1.86	19.2
1021	Inf Monon	340	35.0	103.0	6.5	2.6	1.6	1.30	12.5
971	Cooley's	346	36.0	104.0	4.7	1.2	4.8	1.25	12.0

\* Sample lost in processing

normal range, the unit red blood cell zinc concentration prior to therapy is significantly increased, but returns to within normal limits with successful therapy.

#### DISCUSSION

The over-all limitations of the technique employed (chemical analysis and hematology) have been found to be defined by an error of about  $\pm 15$  per cent.<sup>1</sup>

The direct measurements for whole blood, plasma, red cells and white cells, in

the group of secondary anemias, given in table 1, are graphically depicted in figure 1

TABLE 2.—The Unit Values of Zinc Content of Whole Blood, Plasma, Leukocytes and Erythrocytes in Pernicious Anemia

Exp No	Days of Observation†	RBC per mm. <sup>3</sup>	Hct %	M C V in $\mu^3$	Unit Content by Dithizone Extraction				
					Whole Blood	Plasma	Leukocytes	Erythrocytes	
					$\gamma$ per ml		$\gamma \times 10^{-3}$ per $1 \times 10^6$ cells	$\gamma \times 10^{-3}$ per $1 \times 10^6$ cells	Zn $\gamma$ /cc. packed red cells Corr for M C V
19-1	1	1 80	27 2	152	7 5	1 2	1 6	3 38	22 4
19-2	5	1 94	27 2	143	6 7	1 7	2 6	2 65	19 0
19-3	20	3 70	37 0	100	11 1	4 7	4 1	1 75	17 5
19-4	40	3 23	35 1	108	—*	3 4	7 9	2 35	21 6
19-5	366	3 77	39 7	105	7 0	1 9	1 9	1 49	14 1
21 1	15	2 50	30 3	121	8 7	0 9	19 2	3 10	25 6
21 2	22	2 78	32 3	116	8 6	1 4	1 1	3 44	29 6
21 3	30	2 96	34 1	115	9 3	1 6	4 7	2 82	26 1
21 4	369	4 35	45 1	104	10 6	4 2	2 5	1 69	17 1
21 5	369	4 54	45 0	99	9 3	3 6	2 6	1 72	17 7
57 1	—1	1 22	15 0	123	5 8	2 4	5 3	3 14	23 7
57 2	6	1 59	21 9	131	10 0	3 8	15 0	3 55	29 7
57 3	13	2 20	26 2	115	10 0	3 1	2 8	2 30	21 0
57 5	22	2 48	29 0	117	—*	2 1	2 6	2 78	23 8
57-6	27	2 94	31 5	107	6 4	1 5	2 5	2 52	24 1
57-7	41	3 66	35 2	97	10 5	7 2	—*	2 10	21 0
57 8	55	3 88	37 5	97	5 6	1 1	4 9	1 60	15 9
57 9	208	4 41	44 8	101	7 2	2 0	2 5	1 80	18 7
57 10	257	4 33	43 0	99	8 0	2 0	3 2	1 21	12 3
91 1	—7	2 47	27 2	110	9 3	3 1	3 0	2 80	25 4
91 2	—1	2 43	26 5	109	8 1	2 8	2 2	2 47	22 2
104 1	11	2 60	25 7	99	6 7	1 9	3 6	2 64	26 7
125 1	4	1 59	22 0	138	6 8	4 2	1 7	2 36	17 1
128 1	—5	0 94	14 3	153	5 8	2 4	0 8	3 50	23 0
128-2	1	1 00	14 7	147	10 4	3 7	2 5	4 95	34 0
128-3	8	1 21	19 0	157	5 3	0 7	—*	7 25	46 0
128 4	16	2 07	26 5	128	6 5	0 7	—*	3 67	30 0
128-5	23	2 35	32 0	136	9 6	3 0	1 6	4 05	29 3
128-6	66	4 28	41 0	96	7 5	1 2	1 4	1 59	16 5
128 7	80	4 87	41 0	84	9 0	3 2	1 2	1 41	16 8
129-1	—1	1 42	15 6	110	4 0	1 2	—*	2 24	20 4
132 1	8	1 82	23 3	128	8 6	2 4	—*	2 53	20 0
132 2	15	2 76	26 5	96	7 0	2 1	2 2	2 96	30 7
132 3	69	4 92	44 0	89	6 4	1 0	1 3	1 42	15 9

\* Sample lost in processing

† With reference to institution of therapy

Seventeen of the 23 whole blood zinc measurements (fig 1A) are shown to fall within  $\pm 2$  standard deviations of the mean of our normal series. Case 49 had a

high whole blood zinc due to an increased plasma zinc, while the red blood cell zinc was normal. Case 14 was refractory to all therapy. The whole blood zinc was just above the range of the normal series though within the possible technical limit of error. In Case 17, three consecutive samples were consistently below the lower limit of normal. While under observation, no diagnosis was established, and

TABLE 3 — Zinc Content of 1 ml. of Whole Blood Calculated from Unit Values of Plasma, Leukocytes and Erythrocytes in Anemias other than Pernicious Anemia

Exp. No.	Diagnosis	Plasma	Leukocytes	Erythrocytes	Total Zn in 1 ml. of Whole Blood
Column		1	2	3	4
14-2	Refractory Anemia	4.1	0.14	4.9	9.1
17-1	Refractory Anemia	1.2	0.24	1.5	2.9
17-2	Refractory Anemia	1.4	0.61	3.3	5.3
17-3	Refractory Anemia	0.9	0.15	3.7	4.8
18-1	Refractory Anemia	1.8	0.37	6.0	8.2
62-1	Refractory Anemia	2.8	0.52	5.0	8.3
95-1	Refractory Anemia	7.6	0.23	6.7	9.5
81-1	Hemorrhage	1.0	0.04	4.5	5.5
106-1	Hemorrhage	5.8	0.16	6.8	12.8
118-1	Hemorrhage	1.9	0.16	3.7	5.8
153-1	Hemorrhage	2.3	0.15	4.3	6.8
49-1	Notritional	17.8	0.31	5.4	20.2
139-1	Iron Deficiency	0.8	0.07	4.8	5.7
39-1	Uremia	1.9	0.31	4.7	6.9
112-1	Uremia	1.8	0.37	4.8	7.0
98-1	Sickle Cell	2.9	0.65	8.0	11.6
98-2	Sickle Cell	0.9	—*	5.8	—*
133-1	Sickle Cell	2.9	0.18	6.3	9.4
56-1	Splenic	3.1	0.14	6.1	9.3
66-1	Infection	2.8	0.32	7.7	11.0
107-1	Infection	2.7	0.39	7.1	10.2
102-1	Inf. Moon.	1.7	0.19	4.4	6.3
97-1	Cooley's	0.8	0.19	4.3	5.3

\* Sample lost in processing

Explanation of Columns

1. Zo per ml. of plasma  $\times 100$  — Hematocrit of Whole Blood.

2. Total Zinc in Sample  $\div$  ml. of Whole Blood from which leukocytes obtained.

3. Zo per million red cells  $\times$  red cell count of Whole Blood  $\times 1 \times 10^6$

4. Sum of Columns 1, 2, and 3

we are, therefore, because of the singularity of the observation, unable to draw conclusions from these data.

Figure 1B shows the plasma zinc concentrations, all of which are within the limits of normality except Case 49, previously referred to, and Case 106, for no apparent cause.

Figure 1C is a plot of leukocyte zinc content in terms of  $1 \times 10^{-6}$  gamma per million leukocytes. It is apparent that Cases 17, 18 and 62 have a leukocyte zinc

concentration elevated from three to ten times above normal. These patients were considered refractory to all therapy. They all had leukocyte counts below

TABLE 4.—Zinc Content of 1 ml of Whole Blood Calculated from Unit Values of Plasma, Leukocytes and Erythrocytes in Pernicious Anemia

Exp No	Days of Observation†	Plasma	Leukocytes	Erythrocytes	Total Zn in 1 ml of Whole Blood
Column		1	2	3	4
19-1	1	0.9	0.21	6.1	7.2
19-2	5	1.3	0.26	5.2	6.8
19-3	20	3.0	0.44	6.5	9.9
19-4	40	2.2	0.51	7.6	10.3
19-5	366	1.2	0.15	5.6	7.0
21-1	15	0.6	0.66	7.8	9.1
21-2	22	1.0	0.09	9.6	10.7
21-3	30	1.0	0.50	8.4	10.4
21-4	369	2.3	0.26	7.7	10.3
21-5	369	2.0	0.20	8.0	10.2
57-1	-1	2.0	0.21	3.8	6.0
57-2	6	3.0	0.43	5.7	9.1
57-3	13	2.3	0.14	5.1	7.5
57-5	22	1.5	0.14	6.9	8.5
57-6	27	1.0	0.10	7.4	8.5
57-7	41	4.7	—*	7.7	12.4
57-8	55	0.8	0.24	6.2	7.2
57-9	208	1.1	0.13	8.0	9.2
57-10	257	1.1	0.14	5.2	6.5
91-1	-7	2.3	0.13	6.9	9.3
91-2	-1	2.0	0.12	5.9	8.0
104-1	11	1.4	0.11	6.9	8.4
125-1	4	3.2	0.11	3.8	7.1
128-1	-5	2.1	0.03	3.3	5.4
128-2	1	3.1	0.08	5.2	8.2
128-3	8	0.6	—*	8.7	—*
128-4	16	0.5	—*	7.7	—*
128-5	23	2.0	0.09	9.4	11.5
128-6	66	0.7	0.17	6.8	7.7
128-7	80	1.8	0.08	6.9	8.8
129-1	-1	1.1	0.07	3.1	4.3
132-1	8	1.8	—*	4.6	—*
132-2	15	1.5	0.15	8.2	9.8
132-3	69	0.6	0.09	7.0	7.7

\* Sample lost in processing

† With reference to institution of therapy

Explanation of columns the same as in table 3

2000/mm<sup>3</sup>. This was in contrast to Case 95, which was also found refractory to therapy but had a normal zinc concentration of leukocytes which numbered 12,750/mm<sup>3</sup>. We are not at present prepared to interpret the significance and

physiologic implications of these findings which, however, appear striking in contrast to our findings in leukemic cells. In myelogenous and lymphatic leukemia, the zinc concentration of the circulating leukocytes was found to be about 10 per

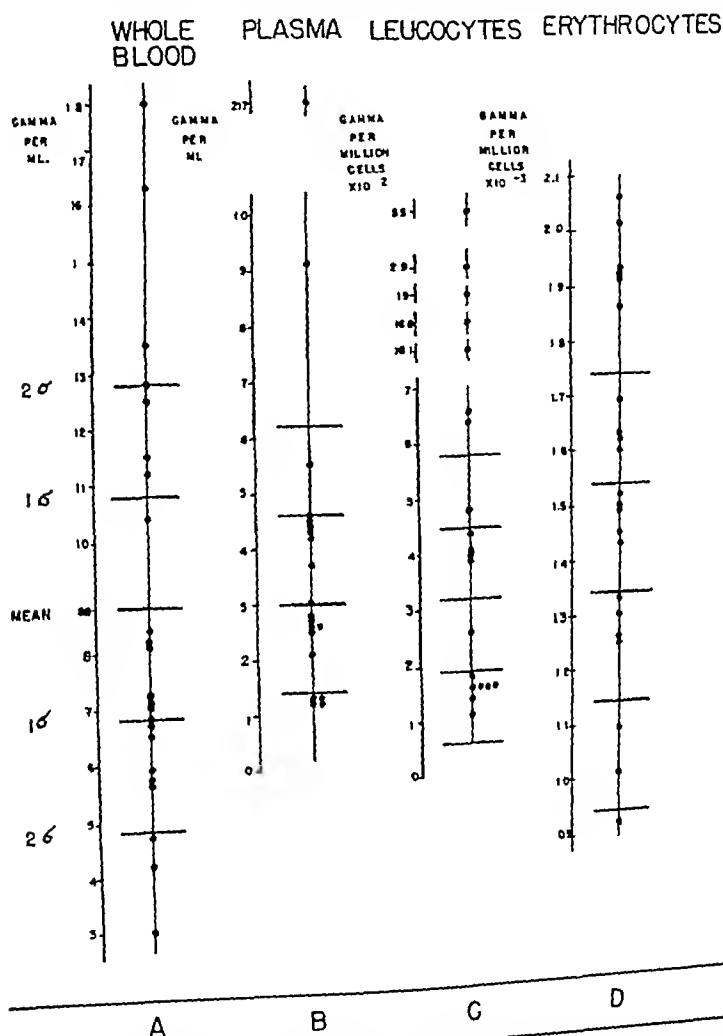


FIG 1—Distribution of zinc in whole blood, plasma, leukocytes and erythrocytes in patients with anemia other than pernicious anemia. The mean and 2 standard deviations of the comparable normal values are indicated.

cent of normal. Under effective therapy with x ray and urethane the zinc concentration returned to normal levels. No other significant abnormalities in the leukocyte picture are apparent.

Figure 1D depicts the red blood cell pattern in these cases of anemia in relation to our normal series. Cases 95 (refractory anemia) and 98 (sickle cell anemia) show significant elevations above the normal. Cases 106, 133, 66 and 107 have zinc concentrations just above the upper limit of the normal distribution curve. It would require a larger number of observations to determine whether or not this is significant or due to chance scattering. With the exception of these cases, there were no abnormalities in the red cell series of this group.

The changes in zinc concentration observed in pernicious anemia patients (table 2) are apparently due to a marked increase in unit zinc content of erythrocytes which are reflected in the whole blood zinc. Neither plasma zinc nor white blood cell zinc presents any significant deviation from the normal distribution picture. The zinc of leukocytes in samples 21-1 and 57-2 was elevated, due to known technical error. Other leukocyte zinc values were within normal limits (fig. 2A, B, C).

The unit red cell zinc concentrations (calculated per million cells (fig. 2D) and per cc. of packed cells) of untreated patients are elevated significantly above the normal range. Following institution of therapy and after an initial rise, the unit zinc content falls successively over a prolonged period. In Cases 57 and 128, samples were obtained at close intervals. On the 55th and 66th days of sampling, respectively, following institution of therapy, the erythrocyte zinc concentration returned to normal levels, and remained normal thereafter under maintenance therapy. In Case 132, normal levels were obtained within 69 days following institution of therapy. In Case 19, no samples were obtainable between the 40th and 366th days of therapy. Similarly, no samples could be obtained between the 30th and 369th days post-therapy in Case 21. While the earlier samples still showed a definitely increased zinc concentration, samples taken 366 and 369 days after treatment was begun were normal in unit erythrocyte zinc content.

Figures 3, 4 and 5 show the erythrocyte zinc concentration per million cells, the red blood cell count, the per cent reticulocytes and absolute number of reticulocytes, in one ml. of blood as a function of time. Doses of liver therapy, given while under hospital care, are indicated by arrows at the top of each graph.

Case 19 in figure 3 shows a prompt reticulocytosis in response to a test dose of liver. The reticulocyte response which is also shown in terms of the absolute reticulocyte count was accompanied by a prompt rise in red blood cell count which continued when the reticulocyte response subsided. During this period there was a progressive fall in the zinc concentration of red blood cells which eventually reached normal levels. Maintenance therapy had no visible effect on the zinc concentration.

The features of Case 57 (fig. 4) were essentially similar to those just described for figure 3. Therapy was spaced very nearly identically. There was a slight rise in zinc concentration at the height of reticulocytosis. However, this rise is entirely within the limits of technical error. The unit zinc concentration fell to normal levels within 55 days following institution of liver therapy.

Case 127 (fig. 5) differs materially in both character and degree of response from these two instances. Coincidental with therapy, observations on this patient to evaluate the potency of the liver extract used were made. The patient received 1 ml. on 1/14/48, with a subsequent submaximal reticulocyte response. The first



zinc sample was obtained near the height of this reaction. Thereafter, intensive daily therapy with the extract was instituted. This resulted in a good reticulocyte response which became maximal on the 20th day following the first liver injection.

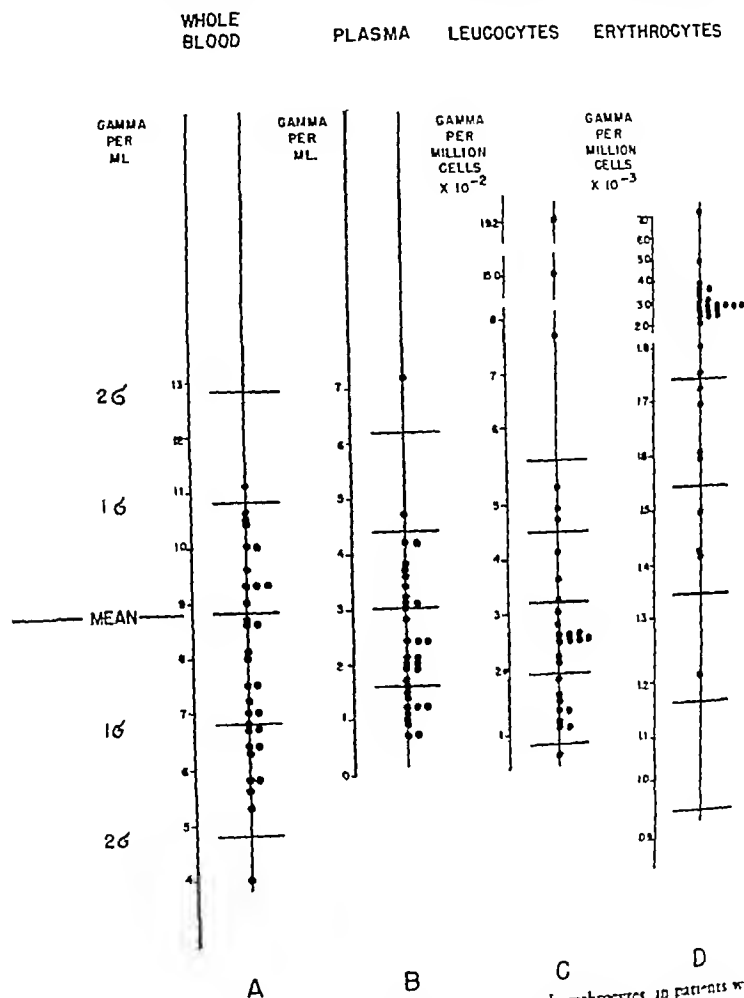


FIG. 2.—Distribution of zinc in whole blood, plasma, leukocytes and erythrocytes in patients with pernicious anemia. The mean and 2 standard deviations of the comparable normal values are indicated.

A prompt rise in total red blood cell count accompanied this phenomenon. The zinc concentration per million cells during this period rose progressively and reached a peak at the height of reticulocytosis and fell concomitantly with it. Thereafter, the zinc concentrations fell to normal levels in a fashion similar to the

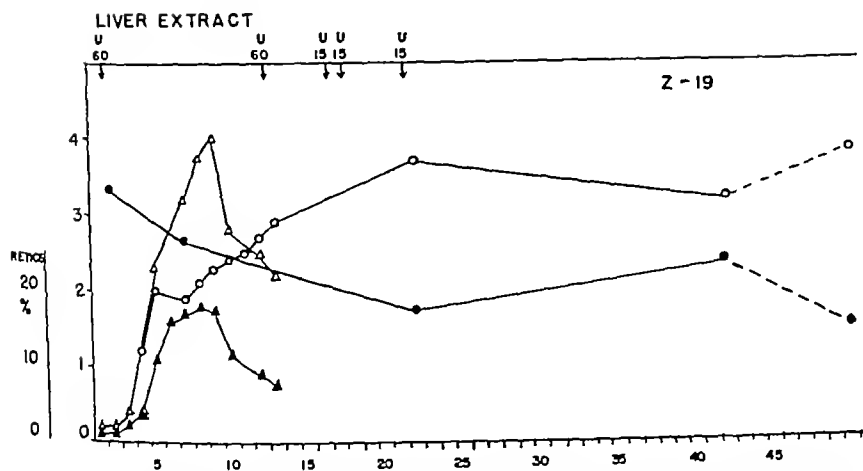


FIG 3—Case 19 Erythrocyte zinc concentration red blood cell count, per cent reticulocytes and absolute number of reticulocytes as well as absolute number of unreticulated erythrocytes, as a function of time under liver therapy

Solid circles = Erythrocyte Zinc concentration per million cells

Open circles = Red Blood Cell count

Solid triangles = Per cent reticulocyte count

Open triangles = Absolute number of reticulocytes

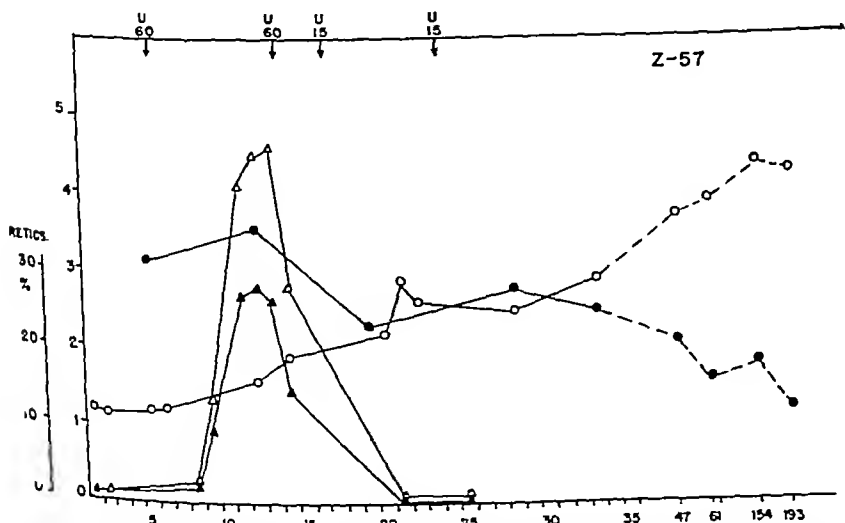


FIG 4—Case 57 Erythrocyte zinc concentration red blood cell count per cent reticulocytes and absolute number of reticulocytes as well as absolute number of unreticulated erythrocytes as a function of time under liver therapy Symbols as in figure 3

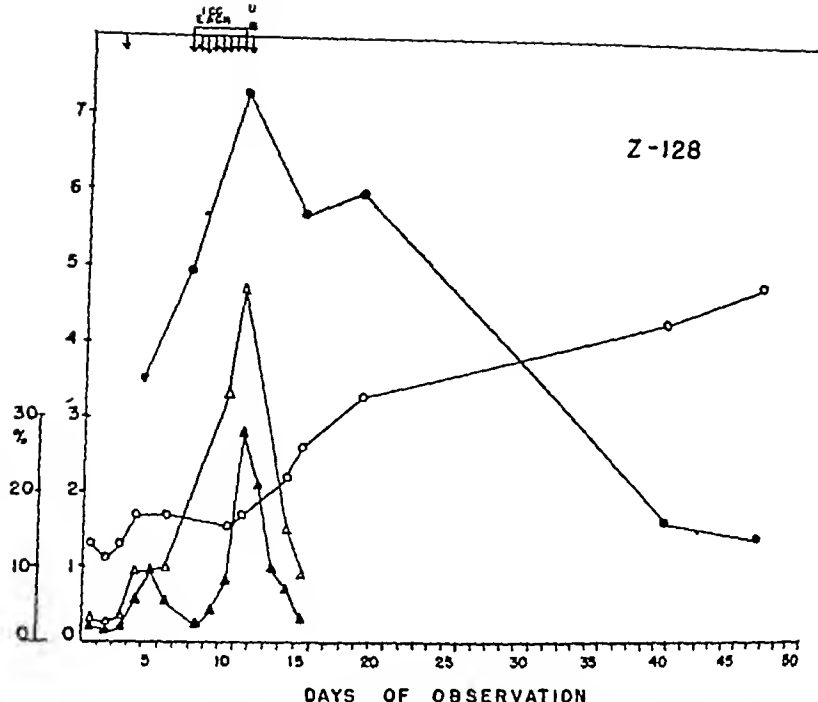


FIG 5—Case 123 Erythrocyte zinc concentration red blood cell count, per cent reticulocytes and absolute number of reticulocytes, as well as absolute number of unreticulated erythrocytes as a function of time, under liver therapy Symbols as in figure 3

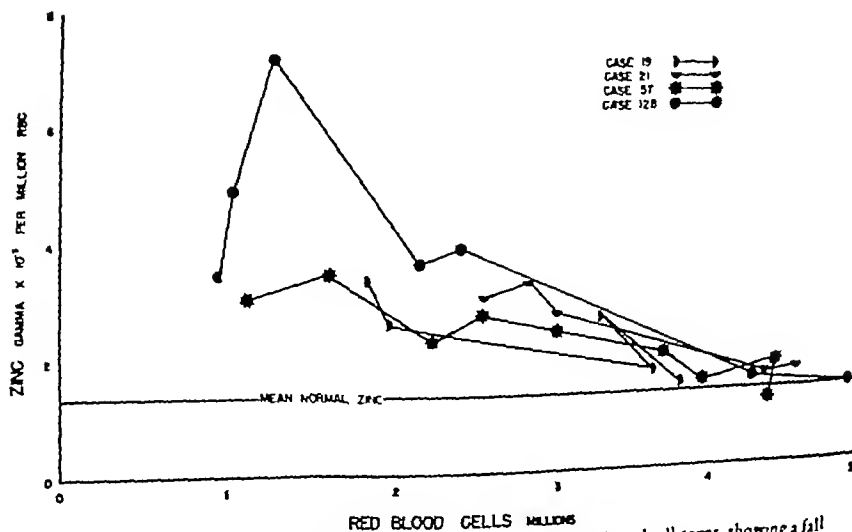


FIG 6—Zinc, in gamma  $\times 10^{-3}$  per million red cells, in relation to the red cell count, showing a fall in 4 patients with pernicious anemia under therapy

ones shown in Cases 19 and 57. The normal level was attained within 66 days. In Case 131, where only three samples were obtained, a normal value was found on the 69th day post-therapy.

Whether or not this response of the zinc concentration was related to the particular liver extract used, the dosage given, or whether it is a property of young cells released from the bone marrow in pernicious anemia under the stimulus of liver therapy, we are unable to conclude on the basis of our present observations.

In the 3 cases we were able to follow, the erythrocyte zinc concentration returned to normal levels within 55, 66 and 69 days respectively. This time closely approximates the mean life span of erythrocytes in pernicious anemia as determined with  $N^{15}$  labelled glycine by London, Shemin and Rittenberg.<sup>8</sup> We believe that the change in zinc concentration is probably a function of the replacement of pernicious anemia cells by cells formed under the influence of liver extract, and in effect is an expression of the death rate of the pernicious anemia cell.

Figure 6 shows the unit zinc concentration in red blood cells in micrograms per million cells, in relation to the red blood cell count. The unit zinc concentration reaches the normal level at the time when the red blood cell count has returned to normal, coinciding with a return of the M C V toward normal size. In the secondary anemias, the zinc concentration per unit cells remains normal in spite of low red blood counts.

Red blood cell zinc was also calculated per cc of packed cells. Thereby the increased size of the individual cell was eliminated as a factor which could contribute to the increased zinc concentration of red cells per million cells. A definite increase over the normal zinc concentration is nevertheless noticed. The phenomenon is therefore not simply one of increased cellular mass.

In anemias other than pernicious anemia, zinc and carbonic anhydrase decrease on a slope parallel to that of the drop in hemoglobin. In contrast, in pernicious anemia, the increase of zinc and carbonic anhydrase are inversely proportional to the fall in hemoglobin indicating that the hemoglobin and carbonic anhydrase systems are structurally discrete though functionally related.

In sickle cell anemia, the zinc concentration of erythrocytes was markedly elevated in one out of three samples on 2 patients. The other two samples were above two standard deviations from the normal mean but so close that they could not be thought to be statistically significant. More observations in this condition are required before a definitive statement about zinc distribution can be made.

Zinc is known to be an integral component of the enzyme carbonic anhydrase.<sup>9, 10</sup> The functional significance of this enzyme in the anemias has recently been scrutinized,<sup>11</sup> and the quantitative relationship of zinc and enzyme activity has been determined independently on samples of venous blood, and is being reported in two other communications.<sup>12, 13</sup>

#### CONCLUSIONS

1. The zinc content of whole blood, plasma, leukocytes and erythrocytes was determined in 20 patients with miscellaneous anemias and in 9 patients with pernicious anemia.

2 Unit values for erythrocytes (zinc in gamma per million cells or per cc of packed cells) were within the limits of normality in the anemias, other than pernicious anemia

3 In pernicious anemia, unit values for erythrocytes were significantly elevated above normal. Under successful liver therapy there was a progressive fall in unit value which reached normal when the red blood cell count had risen to normal

4 The rate of decrease in unit zinc value of the circulating red cells in pernicious anemia is comparable to the probable death rate of cells in circulation prior to the institution of therapy

#### REFERENCES

- <sup>1</sup> VALLEE, B. L. AND GIBSON, J. G., 2ND. The zinc content of normal human whole blood plasma, leukocytes and erythrocytes. *J Biol Chem* 176 445 1948
- <sup>2</sup> SUTTON, W. R. AND NELSON, V. E. Growth reproduction and blood changes produced in rats by means of zinc. *Proc Iowa Acad Sc* 44 117, 1937
- <sup>3</sup> ——— AND ———. Studies on zinc. *Proc Soc Exper Biol & Med* 36 211, 1937
- <sup>4</sup> SMITH, S. E. AND LARSON, E. J. Zinc toxicity in rats. Antagonistic effects of copper and liver. *J Biol Chem* 163 29 1946
- <sup>5</sup> VALLEE, B. L., HUGHES, W. L. JR. AND GIBSON, J. G., 2ND. A method for the separation of leukocytes from whole blood by flotation on serum albumin. *Blood Special Issue* 1 82, 1947
- <sup>6</sup> ———, AND GIBSON, J. G., 2ND. An improved dithizone technique for the analysis of zinc in blood. *J Biol Chem* 176 435 1948
- <sup>7</sup> GIBSON, J. G., 2ND, VALLEE, B. L., FLUHRARTY, R. G., AND NELSON, J. E. Studies of the zinc content of the leukocytes in myelogenous leukemia. *Program of the Fourth International Cancer Research Congress*, Sept. 2-7 1947, Page 104 (To be published in the Proceedings of the Fourth International Cancer Research Congress)
- <sup>8</sup> LONDON, I. M., SHEMIN, D., AND RITTENBERG, D. The application of the isotope technique to the study of the rates of formation of blood constituents in man. *J Clin Investigation* 26 1188 1947
- <sup>9</sup> KEILIN, D. AND MANN, T. Carbonic anhydrase purification and nature of the enzyme. *Biochem J* 34 1163 1940
- <sup>10</sup> HOVE, E., ELVERHJEM, C. A. AND HART, E. B. The relation of zinc to carbonic anhydrase. *J Biol Chem* 136 425 1940
- <sup>11</sup> LEWIS, H. D. AND ALTSCHULE, M. D. Blood carbonic anhydrase activity in anemia with a note on polycythemia vera. *Blood this issue* p 442
- <sup>12</sup> VALLEE, B. L., LEWIS, H. D., ALTSCHULE, M. D. AND GIBSON, J. G., 2ND. The relationship between carbonic anhydrase activity and zinc concentration of erythrocytes in normal in anemic and in other pathologic states. *Blood this issue* p 467
- <sup>13</sup> ———. Zinc and carbonic anhydrase content of red cells in normals and in pernicious anemia. *J Clin Investigation* 27 559 1948

# THE RELATIONSHIP BETWEEN CARBONIC ANHYDRASE ACTIVITY AND ZINC CONTENT OF ERYTHROCYTES IN NORMAL, IN ANEMIC AND OTHER PATHOLOGIC CONDITIONS

By BERT L. VALLEE, M D , HERBERT D. LEWIS, M D , MARK D. ALTSCHULE, M D ,  
AND JOHN G. GIBSON, II, M D

IT HAS been shown that zinc is a component of the carbonic anhydrase molecule.<sup>1, 2</sup> This enzyme has been demonstrated to catalyze the reaction  $\text{H}_2\text{O} + \text{CO}_2 \rightleftharpoons \text{H}_2\text{CO}_3$  in vitro, its molecular weight is approximately half that of hemoglobin,<sup>3</sup> and some of its other physico chemical properties are established.<sup>3, 4</sup> Carbonic anhydrase obtained from ox and sheep blood has been found to contain 31-34 per cent of zinc per unit of dry, active protein, these values having been obtained by means of a diphenylthiocarbazonate method.<sup>1, 2</sup> This method, when applied to human carbonic anhydrase, indicated the presence of 0.164 per cent of zinc, according to Keilin and Mann.<sup>1</sup> Other investigators, using an older and less sensitive technique,<sup>5</sup> reported ox carbonic anhydrase to contain 0.20 to 0.23 per cent of zinc,<sup>6</sup> and later confirmed these results by polarography.<sup>7</sup>

The nature of the bond between the metal and protein, and the chemistry of the enzyme are not understood at present. The enzyme may be inactivated by various substances. Acids separate zinc from its proteinous prosthetic group, this process is irreversible, a finding which led Keilin and Mann<sup>1</sup> to consider the metal to be the active part of the enzyme molecule. When the enzyme is inactivated by long standing, or by manipulation, zinc remains bound to the protein and cannot be removed by dialysis. While the separation of zinc from enzyme protein irreversibly destroys its activity, the inactivation of the enzyme does not necessarily liberate the metal.<sup>1</sup> Under some circumstances, the presence of an excess of zinc may be due, therefore, to the presence of inactivated enzyme retaining its full complement of zinc.

Zinc is found in human plasma, erythrocytes and leukocytes,<sup>8, 14</sup> but carbonic anhydrase activity can be detected in the blood only in erythrocytes.<sup>15</sup> The carbonic anhydrase activity and zinc concentration, determined separately in normal and pathologic human erythrocytes, have been found to vary within relatively narrow limits.<sup>11, 14, 16</sup> The present study was made to investigate the possible correlation of these two erythrocyte parameters in normal and abnormal states of health.

## METHOD

Venous blood samples were obtained as previously described.<sup>17</sup> Aliquots of each sample were studied for their carbonic anhydrase activity and zinc concentration. The enzyme measurement was performed on

From the Medical Clinic Peter Bent Brigham Hospital the Medical Service and Medical Research Laboratories Beth Israel Hospital and the Department of Medicine Harvard Medical School Boston Mass

Part of this work was aided by a grant from the National Institute of Health the remainder was performed in accordance with a contract between Harvard University and the Office of Naval Research U S Navy

whole blood according to the method described by Altschule and Lewis<sup>16</sup>, a large number of control measurements showed no activity in either plasma or leukocytes.<sup>16</sup> The activities found were, therefore equivalent to the carbonic anhydrase content of the red cell mass contained in 1 cc. of whole blood. An attempt was made to keep the interval between venipuncture and analysis at a minimum to avoid inactivation of the enzyme by excessive manipulation or changes in temperature. Measurements of zinc and blood counts were made according to the routine previously employed.<sup>17</sup>

For statistical purposes the normal series was separated into male and female subjects but the other groups were not.

Data for zinc (Zn) and carbonic anhydrase (U\*) were expressed in terms of their relative quantities in

- (1) The erythrocytes in 1 cc. of whole blood
- (2) each million of red blood cells
- (3) each cc. of packed cells and
- (4) in relation to each gm. of hemoglobin

Finally, the ratio  $R = \frac{\text{Zn/cc. packed cells}}{\text{U/cc. packed cells}}$  was calculated as an index of the interdependence of the two functions investigated.

In the normal and leukemic series the number of cases allowed of an internal statistical analysis. In the remaining groups the data have been compared to the normal mean  $\pm 2\sigma$  the value which includes at least 95 per cent of the observations. The factor  $R = \frac{\text{Zn}}{\text{U}}$  was treated statistically in all five groups.

### MATERIAL

A total of 103 samples of blood obtained from 77 individuals was studied. Twenty-eight samples were obtained from 13 male and 12 female medical students and technicians who comprised the normal group. Eighteen samples from 15 subjects who had leukemia or malignant diseases of the lymphatic tissues were analyzed. Twenty-seven samples were obtained from 10 patients with pernicious anemia, 9 of these were either untreated or had been treated with liver for a very short period when first seen. Two of the former were followed for periods of from 10 to 369 days while one had been under liver maintenance therapy for one year.

Three patients had refractory anemia. Four patients had hemianemic as a consequence of hemorrhage, 2 of these patients had ceased bleeding when studied and had restored their hematological picture to normal levels. Three samples were obtained from 2 patients with sickle cell anemia. Two patients had anemia of infection, and one each had nutritional, iron deficiency and Cooley's anemia.

Four samples from 3 patients with polycythemia secondary to pulmonary fibrosis and emphysema were examined. Three other patients had polycythemia vera, one each had infectious mononucleosis, congestive failure, acute rheumatic fever, jaundice secondary to carcinoma of the head of the pancreas, hemochromatosis, cirrhosis and multiple sclerosis.

The age of the subjects in the normal and pathologic series ranged from 20 to 83 years.

### OBSERVATIONS

In normal subjects, the absolute values for zinc concentration and for carbonic anhydrase activity, and the values for these functions relative to the various hematologic measurements were within the normal range previously reported,<sup>11, 15</sup> the means and standard deviations found in the present study did not differ significantly from those of the other series.<sup>11, 15</sup> There was no difference between the sexes. The value for R for all normal subjects was  $4.1 \pm 0.98$  under the conditions of the present study, and the correlation between zinc and carbonic anhydrase levels was good (table 1, fig. 1). Actually, zinc and carbonic anhydrase measurements paralleled each other closely regardless of the presence or absence, or the nature of pathologic change (tables 1-5). The Pearson method moment coefficient

\* U = Units as defined by Altschule and Lewis

of correlation between the two measurements was 0.48 for all 103 samples, a numerical value of 0.48 represents good correlation. The ratio  $R$  varied within narrow limits close to the normal.

TABLE 1—Zinc and Carbonic Anhydrase Levels in Normal Human Red Cells

No	Sex	MCV		RBC		Hct		Hgb		Zn due to RBC in Whole Blood	U	Zn $\gamma$ $1 \times 10^{-3}$ per million cells	U† $1 \times 10^{-10}$ per million cells	Zn $\gamma$ /cc packed cells	U/cc. packed cells	Zn $\gamma$ /Gm Hb	U/Gm Hb	Zn/cc RBC	RBC
		$\mu^2$	per mm <sup>2</sup>	%	Gm	gamma	$1 \times 10^{-1}$	$1 \times 10^{-1}$	R										
23-1	M	91.5	5.08	46.5	16.0	6.2	2.2	1.21	4.3	13.2	4.7	0.39	0.14	2.8					
29-2	M	93.0	4.20	39.2	13.0	6.1	1.9	1.45	3.6	15.3	5.1	0.47	0.16	3.0					
53-1	M	95.6	4.30	44.9	14.7	7.3	1.6	1.69	3.7	14.9	3.3	0.49	0.11	4.5					
73-1	M	100.0	4.53	45.5	15.1	5.5	1.5	1.20	2.8	11.9	3.6	0.36	0.10	3.3					
84-1	M	97.0	4.38	44.8	14.3	5.7	1.4	1.30	3.2	12.7	3.1	0.40	0.10	4.1					
85-1	M	94.0	5.11	48.5	14.3	5.9	1.3	1.15	2.5	12.2	2.7	0.42	0.09	4.5					
88-1	M	99.0	5.23	52.0	17.0	8.9	1.5	1.71	2.9	17.0	2.9	0.52	0.09	5.9					
88-2	M	79.5	6.37	50.0	16.4	7.5	1.3	1.19	2.0	15.2	2.6	0.46	0.08	5.9					
99-1	M	93.5	4.61	43.2	12.6	5.9	2.0	1.28	3.8	13.6	4.3	0.47	0.14	3.2					
108-1	M	93.2	4.85	45.2	14.6	7.7	1.4	1.59	2.9	17.1	3.1	0.53	0.10	5.5					
109-1	M	100.0	4.60	45.8	14.3	8.4	1.9	1.83	4.1	18.3	4.1	0.59	0.13	4.5					
119-1	M	104.0	4.27	44.3	13.5	6.6	2.3	1.54	3.4	14.9	5.2	0.49	0.17	2.9					
122-1	M	98.0	4.75	46.5	13.4	6.1	1.3	1.29	2.7	18.4	2.8	0.40	0.08	6.6					
90-1	M	85.5	5.32	45.5	15.8	6.4	1.7	1.20	3.2	14.1	3.7	0.40	0.11	3.8					
Mean										6.7	1.7	1.38	3.3	14.8	3.7	0.46	0.11	4.3	
S.D										$\pm 1.06$	$\pm 0.33$	$\pm 0.21$	$\pm 0.90$	$\pm 2.17$	$\pm 0.93$	$\pm 0.064$	$\pm 0.030$	$\pm 1.23$	
24-1	F	89.5	4.46	40.0	13.6	5.6	1.9	1.26	4.3	13.8	4.7	0.41	0.14	2.9					
26-1	F	95.0	4.47	42.5	14.6	4.7	1.6	1.06	3.6	11.3	3.8	0.31	0.11	3.0					
82-1	F	96.5	4.56	44.0	14.0	7.4	2.1	1.60	3.8	16.7	4.0	0.52	0.13	4.2					
83-1	F	105.0	4.20	44.3	14.0	6.9	1.8	1.63	3.8	15.6	4.0	0.49	0.13	3.9					
86-1	F	94.0	3.72	35.0	11.8	6.1	1.7	1.63	4.6	17.1	4.2	0.51	0.14	4.1					
86-2	F	99.0	4.03	40.0	12.8	5.6	1.5	1.38	3.7	13.9	3.8	0.43	0.12	3.7					
86-3	F	93.0	4.40	41.0	12.8	6.8	1.6	1.54	3.7	16.5	3.9	0.53	0.13	4.2					
87-1	F	112.0	3.82	43.0	13.2	5.3	1.7	1.42	3.7	12.8	3.2	0.41	0.11	4.0					
93-1	F	90.5	5.08	46.0	14.5	7.8	1.9	1.54	4.3	17.0	4.1	0.54	0.12	4.2					
100-1	F	105.0	3.94	41.3	11.3	4.9	1.8	1.24	4.6	11.9	4.4	0.43	0.16	2.7					
101-1	F	93.5	4.10	43.9	12.8	5.6	1.7	1.32	3.6	14.2	3.7	0.48	0.13	3.8					
115-1	F	91.0	4.83	44.0	13.0	5.7	1.2	1.17	2.3	12.9	2.7	0.43	0.09	4.8					
116-1	F	100.5	4.31	43.5	12.8	7.9	2.2	1.85	5.1	18.4	5.0	0.62	0.17	3.7					
123-1	F	98.0	4.59	45.0	12.4	7.0	1.5	1.52	3.3	15.3	3.3	0.56	0.12	4.7					
Mean										6.3	1.7	1.44	3.9	14.9	3.9	0.48	0.13	3.9	
S.D										$\pm 0.95$	$\pm 0.24$	$\pm 0.23$	$\pm 0.68$	$\pm 1.92$	$\pm 0.58$	$\pm 0.075$	$\pm 0.021$	$\pm 0.65$	
Total Mean										6.5	1.4	1.41	3.6	14.8	3.8	0.47	0.12	4.1	
Total S.D										$\pm 1.00$	$\pm 0.29$	$\pm 0.22$	$\pm 0.81$	$\pm 2.04$	$\pm 0.6$	$\pm 0.070$	$\pm 0.026$	$\pm 0.98$	

U = Units of carbonic anhydrase as defined by Altschule and Lewis.<sup>17</sup>

† This Unit is the mean corpuscular carbonic anhydrase.

In patients with diseases other than pernicious anemia, both the zinc and carbonic anhydrase activity of one ml. of whole blood were found to be above or below the normal level to a degree proportional to the red blood cell count and hemato-



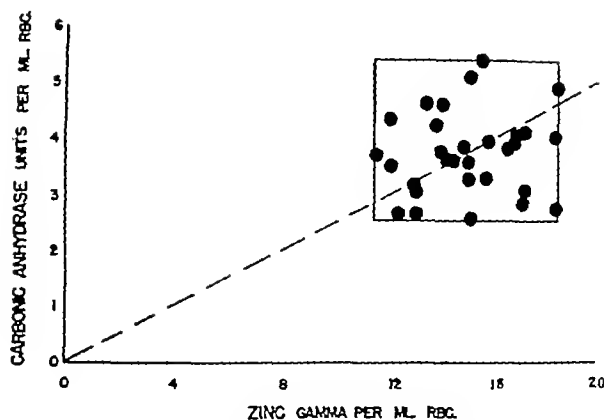


FIG. 1—The relationship of Zn in micrograms per cc. of erythrocytes to carbonic anhydrase in U units per cc. of erythrocytes in normal subjects

The rectangle denotes the normal range. The line connecting with the zero point is drawn through the normal mean of both parameters

TABLE 2—Zinc and Carbonic Anhydrase Levels in Anemias other than Pernicious Anemia

No	Diagnosis	RBC	Hct	MCV	Hgb	Zn due to RBC in Whole Blood	U	Zn <sup>1</sup> X 10 <sup>-3</sup> per million cells	U <sup>1</sup> X 10 <sup>-3</sup> per million cells	Zn <sup>2</sup> γ/cc. packed cells	U <sup>2</sup> /cc. packed cells	Zn <sup>3</sup> γ/Gm Hb	U/Gm Hb	R = $\frac{Zn^{1/cc} RBC}{U^{1/cc} RBC}$
		per mm. <sup>3</sup>	%	μ <sup>3</sup>	Gm	gamma						1 X 10 <sup>-1</sup>	1 X 10 <sup>-1</sup>	
17-2	Refractory Anemia	3.60	31.6	88.0	10.2	3.3	1.1	—	3.1	10.5	3.5	0.33	0.11	3.0
18-1	Refractory Anemia	3.66	36.6	100.0	9.5	6.0	1.5	1.60	4.0	16.4	4.1	0.35	0.16	4.0
62-1	Refractory Anemia	3.75	36.7	90.5	12.3	5.0	2.0	1.33	5.4	15.6	5.4	0.40	0.16	2.5
30-1	Post TRx	5.10	42.8	84.0	14.4	5.9	1.6	1.16	3.1	15.8	3.7	0.41	0.11	3.7
106-1	Hemorrhage	3.52	36.2	88.6	11.4	6.8	1.5	1.92	4.3	18.8	4.1	0.59	0.13	4.4
118-1	Hemorrhage	2.92	24.0	82.2	6.2	3.7	1.3	1.26	4.5	15.4	5.4	0.60	0.21	2.9
170-1	Post TRx	3.32	33.0	90.5	10.3	5.4	2.0	1.34	2.5	15.3	2.9	0.43	0.09	5.1
49-1	Nutritional	4.76	37.5	79.0	10.8	4.8	1.5	1.01	3.2	12.8	4.0	0.44	0.15	3.2
139-1	Iron Def	3.95	31.0	78.0	8.1	8.0	1.8	2.01	4.6	25.8	6.4	0.99	0.22	2.5
98-1	Sickle Cell	3.85	34.0	88.0	8.1	5.8	2.0	1.50	5.2	17.0	5.9	0.72	0.25	2.9
98-2	Sickle Cell	3.29	32.0	97.5	10.2	6.3	1.4	1.91	4.3	19.6	4.4	0.62	0.14	4.5
133-1	Sickle Cell	3.96	39.0	99.0	12.3	7.7	1.8	1.95	4.5	19.8	4.6	0.62	0.15	4.5
66-1	Infection	3.78	36.9	97.5	10.2	7.1	1.3	1.86	3.4	19.2	3.5	0.69	0.11	5.5
107-1	Infection	3.46	36.0	104.0	9.4	4.3	1.7	1.25	4.9	12.0	4.7	0.46	0.18	2.6
97-1	Cooley's													
Mean														3.6
S.D.														±0.99

crit, in almost all instances values of the enzyme and of the metal per ml. of packed erythrocytes were usually within normal limits (tables 1, 2, 4, 5). There were coincidental increases in both zinc and carbonic anhydrase activity per unit of blood

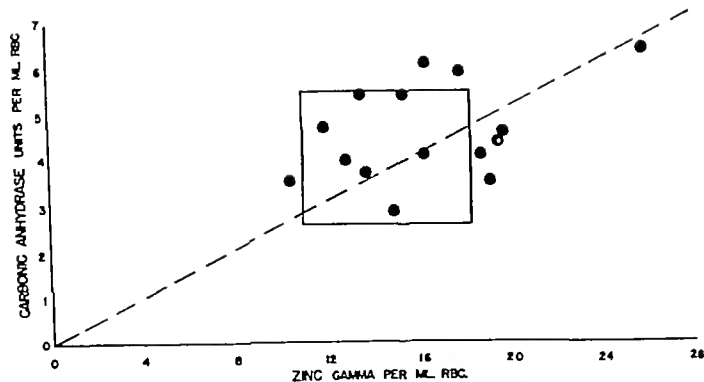


FIG. 2.—The relationship of Zn in micrograms per cc of erythrocytes to carbonic anhydrase in U units per cc of erythrocytes in anemias other than pernicious anemia

The rectangle denotes the normal range. The line connecting with the zero point is drawn through the normal mean of both parameters

TABLE 3 —Zinc and Carbonic Anhydrase Levels in Pernicious Anemia Treated and Untreated

No	Days of Observation	RBC	Hct	MCV	Hgb	Zn due to RBC in Whole Blood	U	Zn $\gamma$ l $\times 10^{-1}$ per million cells	U $\times 10^{-1}$ per million cells	Zn $\gamma$ /cc packed cells	U/cc packed cells	Zn $\gamma$ /Gm Hb	U/Gm Hb	Zn/cc RBC	U/cc RBC
		per mm <sup>3</sup>	%	$\mu^2$	Gm	gamma						$1 \times 10^{-1}$	$1 \times 10^{-1}$	R =	
19-2	5	1.94	27.2	143	8.8	5.2	1.6	2.65	8.2	19.0	5.9	0.59	0.18	3.2	
19-3	20	3.70	37.0	100	11.6	6.5	1.8	1.75	4.9	17.5	4.9	0.56	0.16	3.6	
19-4	40	3.23	35.1	108	11.5	7.6	1.6	2.35	5.0	21.6	4.6	0.66	0.14	4.7	
19-5	366	3.77	39.7	105	9.5	5.6	1.2	1.49	3.2	14.8	3.0	0.56	0.13	4.9	
21-1	15	2.50	30.3	121	9.0	7.8	1.8	3.10	7.2	25.6	5.9	0.86	0.20	4.3	
21-2	22	2.78	32.3	116	9.1	9.6	2.1	3.44	7.5	30.0	6.5	1.07	0.23	4.6	
21-3	30	2.96	34.1	115	8.7	8.4	2.3	2.82	7.8	24.7	6.8	0.97	0.26	3.6	
21-4	369	4.33	45.1	104	12.4	7.7	1.8	1.69	4.1	17.7	4.6	0.62	0.15	4.4	
57-1	—1	1.22	15.0	123	5.5	3.8	1.9	3.14	15.6	25.5	12.7	0.70	0.35	2.0	
57-3	13	2.20	26.2	115	6.2	7.5	1.9	3.43	8.6	28.8	7.2	1.22	0.31	4.0	
57-5	22	2.48	29.0	117	8.1	6.9	2.4	2.78	9.7	23.8	8.3	0.85	0.30	2.9	
57-6	27	2.94	31.5	107	9.4	7.4	2.4	2.52	7.4	24.1	7.6	0.79	0.26	3.2	
57-9	205	4.41	44.8	101	13.9	8.0	1.3	1.80	2.9	17.8	2.9	0.57	0.09	6.1	
57-10	257	4.33	43.0	99	12.6	5.2	1.5	1.21	3.5	12.0	3.5	0.41	0.12	3.4	
91-1	—7	2.47	27.2	110	8.1	6.9	2.1	2.80	8.5	25.2	7.7	0.85	0.20	3.3	
104-1	11	2.60	25.7	99	7.5	6.9	1.3	2.64	5.7	26.9	5.8	0.92	0.20	3.5	
125-1	4	1.59	22.0	138	6.1	3.8	1.1	2.36	6.9	16.8	5.1	0.62	0.17	3.3	
128-1	—5	0.94	14.3	153	4.0	3.3	1.8	3.50	19.2	23.0	12.6	0.83	0.45	1.8	
128-2	1	1.00	14.7	147	3.7	3.0	1.8	4.95	18.0	34.0	12.2	1.34	0.49	2.8	
128-4	16	2.07	26.5	128	6.5	7.7	2.2	3.67	10.6	30.0	8.3	0.85	0.34	3.6	
128-5	23	2.35	32.0	136	9.3	9.5	1.3	4.05	5.5	29.3	4.1	1.02	0.14	7.1	
128-6	66	4.28	41.0	96	12.1	6.8	1.3	1.59	3.0	16.5	3.2	0.56	0.11	5.2	
128-7	80	4.87	41.0	84	12.1	6.9	1.3	1.41	2.8	16.8	3.2	0.57	0.11	5.3	
129-1	—1	1.42	15.6	110	5.8	3.1	1.4	2.24	9.9	20.4	9.0	0.54	0.24	2.3	
132-1	8	1.82	23.3	128	5.9	4.6	1.3	2.53	7.1	20.0	5.6	0.78	0.22	3.6	
132-2	15	2.76	26.5	96	2	8.2	1.4	2.96	5.1	31.0	5.3	1.14	0.19	5.8	
33-1	560	5.28	42.5	81	13.8	8.8	2.3	1.66	4.9	20.7	5.5	0.64	0.15	3.8	
Mean															3.9
S.D.															$\pm 1.2$

With reference to institution of therapy

in Case 98 (table 2, fig. 2) which was an instance of sickle cell anemia. No other cases of anemia in this group show a simultaneous deviation from the normal unit values for zinc and carbonic anhydrase content. The R value in patients with diseases other than pernicious anemia was  $3.6 \pm 0.99$ , the mean being somewhat lower than found in the normal series, but with a similar standard deviation.

The data on patients with pernicious anemia (table 3, fig. 3) show concomitant significant elevation of unit zinc concentrations and carbonic anhydrase activities in the untreated state, with both gradually returning to normal with

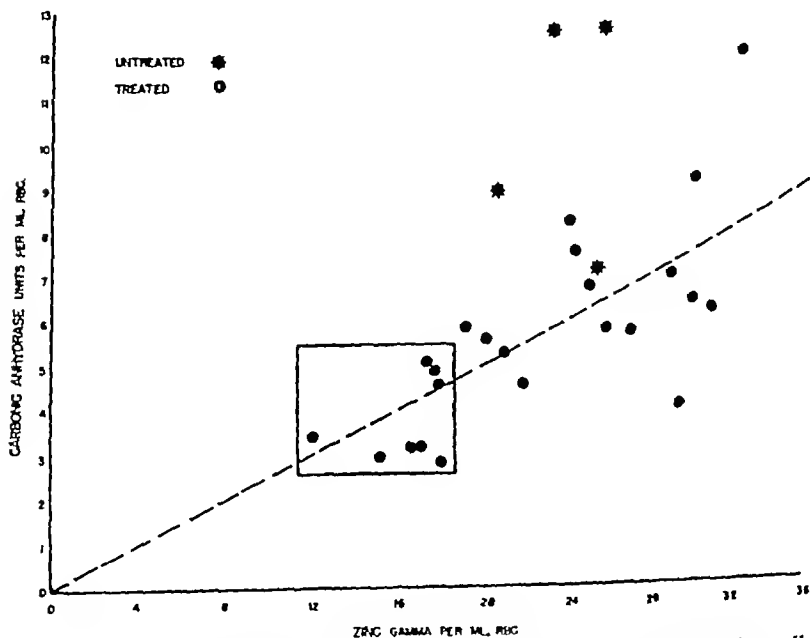


FIG. 3—The relationship of Zn in micrograms per cc. of erythrocytes to carbonic anhydrase in U units per cc. of erythrocytes in pernicious anemia.

The rectangle denotes the normal range. The line connecting with the zero point is drawn through the normal mean of both parameters.

effective therapy. The values for both, in 1 ml. of whole blood, are correspondingly higher than could be expected on the basis of the hematologic findings and, therefore, are normal or nearly normal in the absolute sense.

In all cases of pernicious anemia seen before or shortly after institution of therapy, the red cell zinc concentration and carbonic anhydrase activity per unit of red cells were markedly and concomitantly elevated. Under therapy, both parameters returned to normal levels (fig. 4). In Case 128, where a prolonged follow-up study was possible, there was a simultaneous return of both functions to normal on the 66th day. In the untreated cases, R is lower than normal, suggesting the possibility of a relative increase in carbonic anhydrase in relation to zinc.

It should be pointed out that in anemic blood at activities as high as the ones which these values represent, the bio-assay technic is not as reliable as it is at lower values. However, the possibility cannot be excluded that a true activation of the enzyme was present under these circumstances. The mean R value for the whole series, however, was normal.

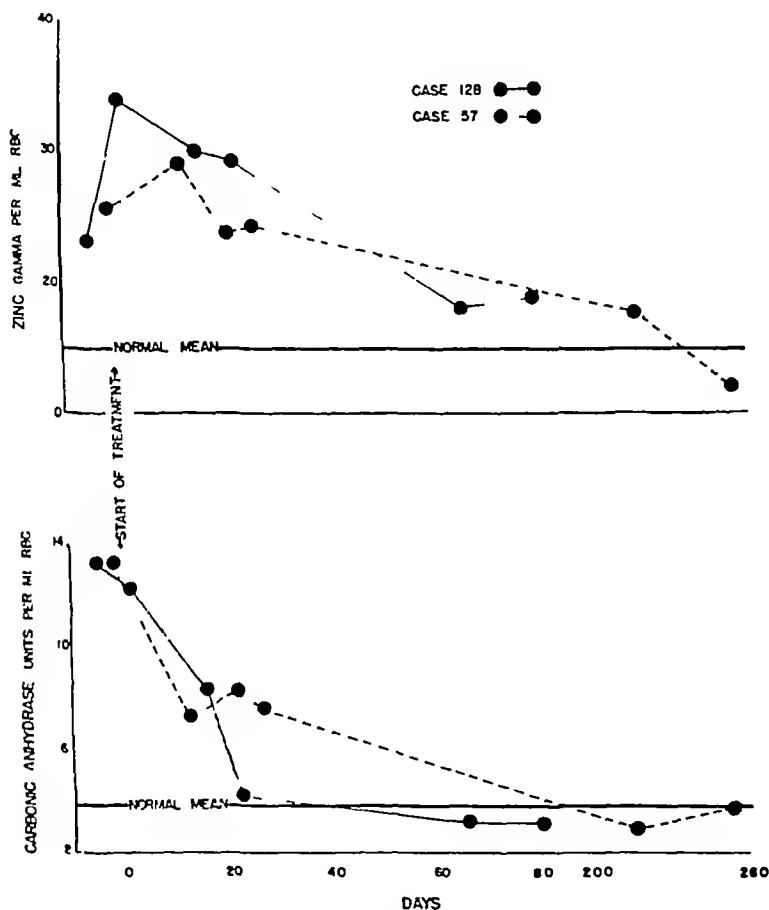


FIG 4—Simultaneous change of Zn content and carbonic anhydrase activity in erythrocytes as a function of days under treatment in two patients with pernicious anemia

In the case of leukemic red blood cells the spread of values was considerable, as indicated by a large standard deviation (table 4, fig 5). However, the mean of both variables was within the normal range. Samples No 10-76, No 44-2, and No 63-2, showed a concomitant rise in unit zinc and U values. There was a rise in zinc, not accompanied by a rise in U in Case No 10-12. R reflects the wide range of measurements though remaining within normal limits.

TABLE 4—Zinc and Carbonic Anhydrase Levels in Leukemia and Associated Conditions

No	Diagnosis	RBC	Zn due to RBC in Whole Blood	U	Zn $\gamma$ X $10^{-3}$ per million cells	U $1 \times 10^{-3}$ per million cells	Zn $\gamma$ /cc packed cells	U/cc. packed cells	Zn $\gamma$ /Gm. Hb	U/Gm. Hb	Zn/cc. RBC U/cc. RBC
		per mm. <sup>3</sup>	gamma						$1 \times 10^{-3}$	$1 \times 10^{-1}$	
10-1	Myelogenous Leukemia	2 60	3 6	0 7	1 37	2 7	18 0	3 4	0 45	0 09	5 3
10-12	Myelogenous Leukemia	2 81	5 5	1 0	1 94	3 6	22 7	4 1	0 62	0 11	5 5
10-76	Myelogenous Leukemia	2 89	6 0	2 0	2 08	7 0	22 2	7 4	0 81	0 27	3 0
22-1	Myelogenous Leukemia	3 06	3 9	1 7	1 27	5 6	14 6	4 8	0 38	0 16	3 0
22-3	Myelogenous Leukemia	3 68	5 9	1 7	1 60	4 6	18 5	5 3	0 55	0 16	3 5
44-2	Myelogenous Leukemia	4 09	8 2	2 3	1 99	5 6	23 2	6 5	0 68	0 19	3 6
57-2	Myelogenous Leukemia	2 88	3 7	1 8	1 27	6 2	12 2	6 0	0 47	0 23	2 0
63-2	Myelogenous Leukemia	3 78	7 5	2 1	1 97	5 6	21 1	6 0	0 63	0 18	3 5
68-1	Lymphatic Leukemia	2 42	3 2	1 1	1 32	4 5	15 1	5 2	0 48	0 17	3 0
94-1	Myelogenous Leukemia	3 17	4 8	1 6	1 52	5 1	16 5	5 3	0 78	0 26	3 1
113-1	Lymphatic Leukemia	3 43	6 7	1 1	1 96	3 2	19 2	3 1	0 66	0 11	6 2
126-1	Myelogenous Leukemia	5 61	8 6	1 5	1 53	2 7	15 5	2 7	0 64	0 11	5 7
35-1	Mycosis Fungoides	4 65	6 1	2 1	1 32	4 5	13 9	4 8	0 42	0 15	2 9
38-1	Lympho-Sarcoma	3 72	4 5	1 7	1 22	4 6	12 0	4 5	0 37	0 14	2 7
110-1	Giant Cell	4 24	7 1	1 5	1 68	3 6	17 7	3 7	0 62	0 13	4 8
138-1	Plasma Cell	2 05	3 4	0 9	1 92	4 4	13 0	3 4	0 29	0 03	3 8
137-1	Multiple Myeloma	3 22	5 0	1 5	1 54	4 7	14 8	4 5	0 39	0 12	3 3
140-1	Hodgkin's Disease	3 44	4 8	1 4	1 38	4 1	13 5	3 9	0 41	0 12	3 5
Mean			5 5	1 5	1 60	4 6	16 8	4 7	0 54	0 15	3 8
S.D.			$\pm 1 62$	$\pm 0 43$	$\pm 0 29$	$\pm 1 13$	$\pm 3 54$	$\pm 1 22$	$\pm 0 145$	$\pm 0 055$	$\pm 1 15$

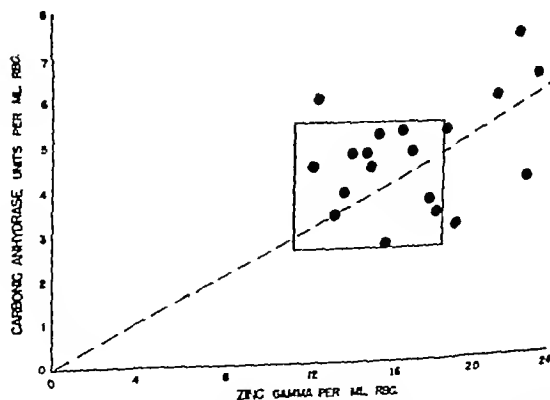


FIG. 5—The relationship of Zn in micrograms per cc of erythrocytes to carbonic anhydrase in U units per cc of erythrocytes in leukemia and allied disorders. The rectangle denotes the normal range. The line connecting with the zero point is drawn through the normal mean of both parameters.

There was no deviation from the normal unit values in polycythemia, the amount of zinc due to red blood cells in 1 cc of whole blood was increased, as a consequence of the increased red blood cell mass. The ratio R was within normal limits.

TABLE 5—Zinc and Carbonic Anhydrase Levels in Miscellaneous Disorders

No	Diagnosis	RBC	Hct	Hgb	Zn due to RBC in Whole Blood	U	Zn $\gamma$ $\times$ $10^{-3}$ per million cells	U $\times$ $10^{-10}$ per million cells	Zn $\gamma$ /cc packed cells	U/cc packed cells	Zn $\gamma$ /Gm Hb	U/Gm Hb	Zn/cc RBC U/cc RBC R
		per mm. <sup>3</sup>	%	Gm	gamma						$1 \times 10^{-1}$	$1 \times 10^{-1}$	
33-1	Pulmonary Fibrosis	5.37	51.8	20.3	10.4	2.2	1.95	4.1	20.2	4.2	0.51	0.11	4.8
33-2	Secondary Polycythemia	5.55	54.7	16.4	9.1	2.0	1.62	3.6	16.5	3.7	0.51	0.12	4.5
105-1	Pulmonary Fibrosis	7.74	75.3	16.3	14.4	2.2	1.85	2.8	18.7	2.9	0.88	0.13	6.4
114-1	Secondary Polycythemia	6.75	55.0	15.5	8.9	1.4	1.30	2.1	16.0	2.5	0.57	0.09	6.4
64-1	Pulmonary Fibrosis	6.55	69.2	23.3	10.0	2.9	1.53	4.1	14.4	3.9	0.43	0.12	3.7
65-1	Polycythemia Vera	6.98	69.2	22.0	9.2	3.1	1.32	4.4	13.3	4.5	0.42	0.14	3.0
136-1	Polycythemia Vera	8.29	60.0	17.2	7.7	2.1	0.93	2.5	12.8	3.5	0.49	0.12	3.7
102-1	Infectious Mononucleosis	3.40	35.0	10.0	4.4	1.7	1.30	5.0	12.6	4.9	0.44	0.17	2.6
50-1	Congestive Failure	5.40	47.0	14.8	7.2	1.9	1.33	3.5	15.2	4.0	0.48	0.13	3.8
71-1	Acute Rheumatic Fever	3.85	36.0	10.6	5.1	1.2	1.33	3.1	14.2	3.3	0.48	0.11	4.3
111-1	Carcinoma of Pancreas	4.20	40.0	11.8	8.7	1.3	2.07	3.1	20.8	3.2	0.74	0.11	6.5
117-1	Hemochromatosis	4.64	51.0	16.2	5.8	1.7	1.24	3.7	11.3	3.2	0.36	0.11	3.5
134-1	Cirrhosis	4.79	44.7	14.9	5.7	1.3	1.18	2.7	12.7	2.9	0.38	0.09	4.4
59-2	Multiple Sclerosis	4.52	44.5	13.7	8.4	2.0	1.86	4.3	18.6	4.5	0.61	0.15	4.1
59-3	Multiple Sclerosis	3.87	39.2	11.9	6.9	1.4	1.78	3.6	17.6	3.8	0.58	0.12	4.6
Mean													4.5
S.D.													$\pm 1.2$

## DISCUSSION

The subjects of the present study on whom concomitant zinc and carbonic anhydrase determinations were carried out have been described in part in previous communications.<sup>11-13</sup> Since it was not possible to obtain simultaneous analyses in all instances in the above series, the present group is a chance sample of the two larger series.

It is clear from the data that both zinc and carbonic anhydrase are present in erythrocytes in a fixed ratio under normal circumstances, and vary simultaneously in disease. Both are independent of hemoglobin concentration, this is apparent in the data on untreated pernicious anemia, where there are normal or high zinc and carbonic anhydrase values in the face of a low hemoglobin. Under therapy, there is a relative fall in zinc and carbonic anhydrase, as opposed to a rising hemoglobin concentration.

It appears from the data that zinc and carbonic anhydrase are mutually dependent variables, their correlation being good in terms of the coefficient of correlation. Absolute correlation did not, however, occur. This may be the consequence of the large error in the method for carbonic anhydrase. Also, both measurements are not analogous, for one is a quantitative chemical technic and the other a method

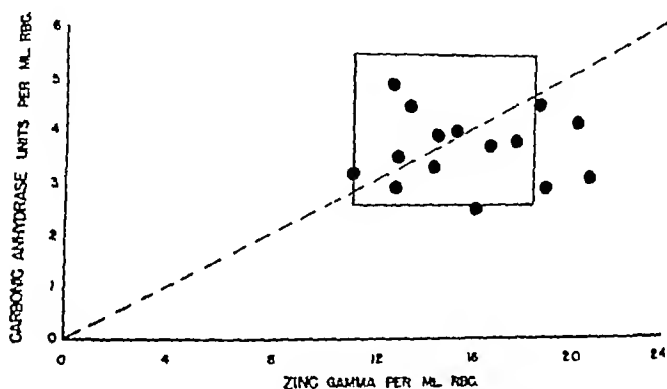


FIG. 6—The relationship of Zn in micrograms per cc. of erythrocytes to carbonic anhydrase in U units per cc. of erythrocytes in miscellaneous disorders (polycythemia vera, secondary polycythemia, infectious mononucleosis, congestive failure, acute rheumatic fever, carcinoma of pancreas, hemochromatosis, cirrhosis, multiple sclerosis).

The rectangle denotes the normal range. The line connecting with the zero point is drawn through the normal mean of both parameters.

measuring biologic activity. Other limitations of the latter procedure have already been pointed out<sup>16</sup>; it should be re-emphasized, moreover, that the conditions for this bio-assay are remote from those which prevail under physiologic circumstances. Measuring the activity of the enzyme under these artificial conditions does not allow quantitative determination of enzyme concentrations. Therefore, the ratio  $R = \frac{\text{Zn/cc packed cells}}{\text{U/cc packed cells}}$  is only an arbitrary coefficient showing the existence of a functional rather than a chemical interrelation. The latter could be established only on a weight by weight basis.

It has been pointed out that the enzyme may be inactivated while retaining its original proportion of zinc. This may possibly account in part for the lack of absolute correlation which has been alluded to. Since there was an inevitable delay

between venipuncture and analysis, a certain amount of manipulation and change in temperature during transport between the two laboratories may also have increased the R value

A change in the degree of physiologic activation of the enzyme cannot be completely dismissed as the possible cause for a decrease of  $R = \frac{Zn}{U}$

Keilin and Mann's data<sup>1</sup> indicate a different zinc concentration for ox and sheep, and human carbonic anhydrase. Because of the facts pointed out above, the present data do not elucidate this point further, since the absolute quantity of enzyme per unit cells cannot be determined with any present technique

It has been suggested<sup>15-18</sup> that carbonic anhydrase deficiency may be an important etiologic factor in dyspnea. It is clear, however, that one cannot correlate with precision the loss of carbonic anhydrase with respiratory or cardiovascular symptoms because of the lack of a method for measurement of the *concentration* of the enzyme. Furthermore, the normal *in vivo* requirements to maintain normal respiratory function are not known at present. With the aid of the here established *in vivo* correlation of concentration of the metal and the activity of the enzyme system, it might become simpler to use erythrocyte zinc concentration as an index of carbonic anhydrase content in some phases of respiratory and humoral physiology

Observations made here demonstrate the occurrence in some anemias of a state of zinc deficiency in the erythrocytes, the findings of the present study indicate the functional significance of this deficiency since the latter is associated with a deficiency of carbonic anhydrase activity. This is strikingly similar to the deficiency of iron in erythrocytes, with its concomitant lowering of hemoglobin level. In addition, it is worthy of note that lack of zinc and of iron in erythrocytes commonly occur together in clinical conditions

#### SUMMARY AND CONCLUSIONS

A good correlation exists between zinc content and carbonic anhydrase activity of the red blood cells under all conditions studied, including anemia and polycythemia. In almost all patients with anemias other than pernicious anemia, both zinc and carbonic anhydrase levels were lowered in parallel fashion. These changes were proportional to decreases in hematocrit and hemoglobin levels and erythrocyte counts so that both zinc and carbonic anhydrase values per unit of RBC were in the normal range. In a few instances of anemia associated with leukemia and in one of sickle cell anemia, neither zinc content nor carbonic anhydrase activity was decreased in proportion to the anemia, in these cases the zinc and carbonic anhydrase levels per unit of blood were both elevated to the same degree.

Patients with pernicious anemia showed no decrease in absolute values for zinc and carbonic anhydrase activity in spite of marked lowering of hematocrit and hemoglobin levels and of erythrocyte count. Accordingly, both zinc concentration and carbonic anhydrase activity per unit of blood were elevated, often to a marked degree. These increases were parallel, varying inversely with the degree of anemia, when they regressed under treatment, both did so at the same rate.



There are no methods available for estimating carbonic anhydrase *concentration*; all methods now in use measure only the *activity* of the enzyme. It is suggested that zinc concentration could be used as an indicator of carbonic anhydrase content of the red blood cells.

#### ACKNOWLEDGMENTS

Drs. Joseph C. Aub and Ira T. Nathanson were kind enough to refer several patients for study. Dr. Byrl J. Kennedy was most helpful in regard to obtaining samples of blood. The technical work was performed by Miss Mary Lou Roney, Betty Hickey and Marion Taylor.

#### REFERENCES

- <sup>1</sup> KEILIN, D., AND MANN, T. Carbonic anhydrase. Purification and nature of the enzyme. *Biochem J* 34<sup>2</sup> 1163, 1940.
- <sup>2</sup> HOVE, E., ELVEHJEM, C. A. AND HART, E. B. The relation of zinc to carbonic anhydrase. *J. Biol. Chem.* 136 425 1940.
- <sup>3</sup> KJESSE, M., AND HASTINGS, A. B. Factors affecting the activity of carbonic anhydrase. *J. Biol. Chem.* 132 281 1940.
- <sup>4</sup> SMITH, E. C. B. Cataphoretic behaviour of carbonic anhydrase. *Biochem J* 34<sup>2</sup> 1176 1940.
- <sup>5</sup> SAHYUN, M., AND FELDKAMP, R. F. The determination of zinc in biological material. *J. Biol. Chem.* 1936 116 555.
- <sup>6</sup> SCOTT, D. A. AND FISHER, A. M. Carbonic anhydrase. *J. Biol. Chem.* 144 371, 1942.
- <sup>7</sup> — AND — Carbonic anhydrase. *Nature* 153 711 1944.
- <sup>8</sup> KEILIN, D. AND MANN, T. Activity of carbonic anhydrase within red blood corpuscles. *Nature* 147 493, 1941.
- <sup>9</sup> VALLEE, B. L. AND FLUHARTY, R. G. Zn metabolism studied by means of the radioactive isotope  $^{65}\text{Zn}$ . *J. Clin. Investigation* 26 1199 1947.
- <sup>10</sup> —, — AND GIBSON, J. G. 2nd. Distribution of zinc in normal blood and organs studied by means of  $^{65}\text{Zn}$ . Program of the Fourth International Cancer Research Congress. Sept. 2-7 1947, Page 137 (To be published in the Proceedings of the Fourth International Cancer Research Congress).
- <sup>11</sup> — AND — The zinc content of normal human whole blood, plasma, leucocytes and erythrocytes (In preparation).
- <sup>12</sup> GIBSON, J. G. 2ND VALLEE, B. L. FLUHARTY, R. G. AND NELSON, J. E. Studies of the zinc content of the leucocytes in myelogenous leukemia. Program of the Fourth International Cancer Research Congress. Sept. 2-7 1947, Page 104. (To be published in the Proceedings of the Fourth International Cancer Research Congress).
- <sup>13</sup> VALLEE, B. L. Zinc and carbonic anhydrase content of red cells in normals and in pernicious anemia. *J. Clin. Investigation* 27 559 1948.
- <sup>14</sup> — AND GIBSON, J. G. 2ND. The zinc content of human whole blood, plasma, leucocytes and erythrocytes in the anemias. *Blood*, this issue, page 455.
- <sup>15</sup> LEWIS, H. D. AND ALTSCHULE, M. D. Blood carbonic anhydrase activity in anemia with a note on polycythemia vera. *Blood*, this issue, page 442.
- <sup>16</sup> ALTSCHULE, M. D. AND LEWIS, H. D. Measurement of carbonic anhydrase activity of blood at body temperature. *J. Biol. Chem.* in press.
- <sup>17</sup> VALLEE, B. L. AND GIBSON, J. G. 2ND. An improved dithizone technique for the analysis of zinc in biologic materials. *J. Biol. Chem.* 176 435 1948.
- <sup>18</sup> ALTSCHULE, M. D. AND LEWIS, H. D. Significance of blood carbonic anhydrase activity in anemia. *J. Clin. Investigation* 27 523 1948.

# HEINZ BODY PHENOMENON IN ERYTHROCYTES

## A REVIEW

By STEWART H WEBSTER, PH D

AMONG the early investigators of hemolytic substances were Casper and Hoppe<sup>5</sup> who in 1859 observed the brown coloration of blood due to nitrobenzene. Since then there has been a continued interest in the action of such toxic materials on the formed elements of the blood. The accelerated development of synthetic organic chemistry during the latter half of the 19th Century created numerous industrial hazards and poisons, at the same time providing the toxicologists with many new compounds with which to work. Among the coal tar products and derivatives of aniline thus produced, phenylhydrazine was of great importance because of its marked physiological action. Prepared in 1875 by Emil Fischer,<sup>25</sup> its behavior in rabbits was studied ten years later by Hoppe-Seyler,<sup>58</sup> the discoverer of methemoglobin.

### EARLY OBSERVATIONS ON MORPHOLOGIC CHANGES IN THE ERYTHROCYTES

The marked action of chlorates had attracted the attention of several workers. As early as 1882, Riess<sup>101</sup> described the presence of one or more small, generally round, globules and granules in the erythrocytes of a person poisoned by potassium chlorate. Drawings were included which showed the appearance of these particles. Somewhat later, Marchand,<sup>85</sup> who had worked on chlorate intoxication previous to this discovery of Riess,<sup>84</sup> observed similar changes in a dog poisoned with the sodium compound. Finally, Lewin<sup>82</sup> had observed the formation of granules within red cells treated *in vitro* with hydroxylamine.

One year later, Robert Heinz (1865-1924), in 1890, studying the action of phenylhydrazine and its derivatives on the blood, observed the changes seen earlier by the above workers, described them in detail, in regard to their appearance, behavior and ultimate fate, and devised a method for staining them, using a wet preparation for this purpose. Drawings were also given showing both the stained and the unstained granules in the blood of several species of animals.<sup>42</sup>

These bodies were depicted by Heinz<sup>42</sup> as round, oval or serrated granules which are very refractile and hence can easily be seen. There may be one or more within the cell wall and they may move around (Brownian motion) or remain fixed in one position. Ordinarily they are eccentrically placed, being located near the margin. Sometimes they appear to protrude from a cell, as if hanging by a stalk, and frequently they can be observed outside the cells in the plasma (schistocytes of Ehrlich). The sizes of the particles vary greatly, being 1-2 microns in diameter in rabbits, guinea pigs and dogs and much larger in cats, often amounting to a third or half of the cell diameter. Heinz recommended supravital examination of the blood, using a dilute solution of methylene blue in isotonic saline solution for staining these

From the Laboratory of Physical Biology, National Institutes of Health, Bethesda, Maryland

particles blue, hence, the term blue particles (blaukörper). This staining solution had been used earlier, in 1882, by Bizzozero<sup>2</sup> in demonstrating the presence of blood platelets.

Initially, Heinz regarded the presence of these granules as pathognomonic of poisoning by phenylhydrazine or its derivatives. However, further researches indicated a large number of substances capable of inducing similar changes. As observed by Heinz, these characteristic changes in the erythrocytes were found in one or more species of animals following administration of many organic compounds such as aniline, toluidine, toluidene diamine, nitrobenzene, dinitrobenzene, p-aminophenol, ethyl aminobenzoate (p), phenylhydrazine, acetylphenylhydrazine and phenylhydroxylamine. However, no such action was observed with benzene, phenol, phenacetin, acetanilid, antipyrine or benzyl amine, and the aliphatic amines. Among inorganic compounds, chlorates and hydroxylamine produced Heinz bodies but not hydrazine or sodium nitrite, although all four substances were very active in producing methemoglobin in vivo.

Ehrlich,<sup>19-21</sup> who at that time was an authority both on anemia and on stain technology, referred to the frequent presence of *hemoglobinemic inclusion bodies* (hämoglobininämische Innenkörper) in erythrocytes of toxic animals as well as in the blood of certain anemic persons. Dustin<sup>17</sup> pointed out that the term inclusion body is unsatisfactory since the particles often appear to be extruded from the cell. The term inner body is likewise objectionable since the particles sometimes appear to be on the surface of the erythrocyte.

Ehrlich and Lindenthal,<sup>18</sup> who found similar bodies in the blood of a person with chronic nitrobenzene poisoning, assigned priority for the discovery of the so-called inner bodies to Ehrlich. This, together with the fact that the senior author's name has been repeatedly misspelled Ehrlich in the literature, presumably has much to do with the confusion which exists even today as evidenced by the use of such numerous terms as Ehrlich-Heinz or Heinz-Ehrlich bodies, Heinz blue granules (Heinzsche Blaukörper), inclusion bodies (Innenkörper or Innenkörperchen), hemoglobinemic inner bodies, hemoglobinemic inclusion bodies, substantia metachromatica granularis,<sup>12</sup>  $\beta$ -substance and Polkörperchen.<sup>20-21</sup>

Heubner,<sup>49</sup> on the basis of Ehrlich's report of 1892,<sup>18</sup> assigned priority to Heinz for this discovery. However, due to the prominence of Ehrlich, many of his ideas prevailed. For example, Ehrlich preferred fixed and stained blood preparations rather than supravital methods. His triacid stain, developed in 1880, was relatively difficult to use, and the results were uncertain with respect to finding inclusion bodies. Heinz preferred the supravital technique but pointed out that both wet and fixed preparations should be used, in order to secure the most information.

Shortly after the initial work of Heinz a number of investigators observed similar intracythrocytic bodies during poisoning with various chemical agents. Thus the earlier findings were confirmed by A. Huber<sup>61</sup> with dinitrobenzene, by Ehrlich and Lindenthal<sup>18</sup> with nitrobenzene, by Schmauch<sup>118</sup> with pyridine, by Schwalbe and Solley<sup>119</sup> with toluidene diamine, by Winogradow<sup>123</sup> with chlorate and by von Domarus<sup>10</sup> with phenylhydrazine. However, much confusion existed in the literature during this period. Thus, Heinz bodies were sometimes identified

with Howell-Jolly bodies, Schmauch found his endoglobular bodies in normal cats, Schwalbe and Solley confused the bodies they saw with blood platelets and found similar forms in normal blood after coagulation. As pointed out by Jurgens and Schürer,<sup>71</sup> marginal bodies (Randkörper), discovered by Röhl in 1890<sup>104</sup> and subsequently rediscovered by Huber, by Schwalbe and Solley, and others, were similar to Heinz bodies in appearance and were often confused with the latter. There was also considerable discussion regarding the identity of Heinz blue granules of the wet preparations with the hemoglobinemic inner bodies seen in Ehrlich's fixed stained preparations. Finally, for many years some workers doubted the existence of Heinz bodies, regarding them as artifacts while others held them to be nuclear debris or nuclear derivatives.

#### CONTRIBUTIONS OF THE SCHOOLS OF PAPPENHEIM, SCHILLING, HEUBNER AND OTHERS

A second period in the development of knowledge regarding the Heinz bodies started about 1911 when Pappenheim and his students were attracted to this field.<sup>94</sup> Almost simultaneously Schilling and his co-workers began similar investigations. Two problems were of paramount importance in the work emanating from these two schools during the following years. The first was concerned with the chemical composition and properties of the Heinz bodies, the other had to do with the mechanism by which they were formed. These problems were so interrelated and so complex that much more data were needed than were then available. A third problem, involving the relationship between methemoglobin- and Heinz body formation, was investigated with a variety of substances by Heubner and his students.<sup>4 38 46 49 51 53 70 76 97 117 122</sup>

#### *Chemical Nature of Heinz Bodies*

Morawitz and Pratt<sup>91</sup> had found that the erythrocytes from animals poisoned by phenylhydrazine were more resistant than normal, as measured with salt solutions and various hemolytic agents. Itami and Pratt<sup>66</sup> proposed the term *pachydermia* to describe this change in resistance of the erythrocyte. They found also that the stroma sediment of anemic blood would not dissolve in water, the particles contained in the residue appearing like Heinz bodies. Sattler,<sup>108</sup> Hirschfeld<sup>86</sup> and Rosenthal<sup>106</sup> all studied the behavior of erythrocytes displaying this increased resistance without arriving at the chemical nature of such a change.

Hartwich,<sup>40</sup> working with Pappenheim, isolated Heinz bodies in large enough quantities to work with them in pure culture. From their solubility in pepsin and hydrochloric acid and from their other reactions it was concluded that the bodies contained protein and lipid material together with some iron.

Pappenheim and Suzuki<sup>95</sup> investigated the behavior of Heinz bodies with respect to their resistance toward certain hemolytic substances, as saponin. Suzuki<sup>123</sup> found that an increase in resistance occurs in blood poisoned *in vivo* by a mixture of pyroline and toluylene diamine, this increase being caused chiefly by the extremely resistant Heinz bodies rather than by a diffuse *pachydermia*.

Further investigations of the composition of these particles were carried out by

Kunkel,<sup>74</sup> who was able to show that they contained no altered hemoglobin but considerable phosphatide, some protein and cholesterol and a colored iron compound which was not identified. Hess and Müller,<sup>47</sup> by selective staining technique, demonstrated that the Heinz particles gave reactions for lipid material.

Heuer<sup>55</sup> drew conclusions somewhat different in that he believed the Heinz bodies to contain neither phosphorus nor phosphoprotein. Later Warburg et al.<sup>119</sup> showed that Heinz bodies produced by phenylhydrazine agreed in behavior and properties with that of denatured globin. More recently, Horecker<sup>59, 60</sup> confirmed this work, using xylydine as the substance for producing the Heinz bodies.<sup>127</sup>

By means of the electron microscope, Jung<sup>68, 69</sup> was able to follow the growth of the Heinz bodies from submicroscopic particles to those attaining half the diameter of the red cells. In the case of dinitroglycol, Heinz bodies were recognizable<sup>66</sup> after a few minutes. Evidence was also obtained that these bodies were at or in the outer surface of the erythrocyte. However, it was found that the structure of the Heinz bodies produced by various hemolytic agents was not identical in all cases. Based on these results and upon their own work, Kiese and Seipelt<sup>73</sup> were led to believe that Heinz bodies contained denatured proteins derived from the erythrocyte membrane.

Finally, as Dustin<sup>17</sup> has pointed out, in the absence of recent comprehensive studies very little can be said with certainty regarding the chemical composition of these particles except that they undoubtedly contain protein material.

#### *Mechanism of Heinz Body Formation*

The formation of Heinz bodies has been explained in a variety of ways, most of which are included in five principal mechanisms.

1 *Protoplasmic theory* Heinz conceived of the blue granules, which were later called by his name, as produced by partial necrosis of the erythrocytic protoplasm. Ehrlich believed these particles to be identical with his hemoglobinemic inclusion bodies, the latter being regarded as containing hemoglobin in a resistant form, either as methemoglobin or as altered hemoglobin. This hypothesis was in agreement with the observation that as the inclusion bodies became larger and more dense, the hemoglobin in the cells appeared to become paler and often disappeared. Since these abnormal structures were produced by substances which simultaneously produced methemoglobin, it was natural to assume that the bodies contained some form of blood pigment. This was supported by the observation that although they were basophilic before fixation, the particles became acidophilic after fixation occurred. However, the work of Kunkel<sup>74</sup> and of Hartwich,<sup>40</sup> in showing that Heinz bodies contain neither hemoglobin nor methemoglobin, appears to dispose of this protoplasmic theory. The observed acidophilic behavior is explained by Gutstein and Wallbach<sup>36</sup> as due to the lipid content of these particles rather than to their hemoglobin content, as believed by Ehrlich.

2 *Nuclear theory* The early attempt to relate Heinz bodies and nuclear remains, such as Howell-Jolly bodies, met with little success. The latter show intense basophilia after fixation while Heinz bodies do not. Zadek and Burg<sup>126</sup> held that no confusion existed in identifying these particles although Schilling<sup>118</sup> maintained that he had observed a transition between these two forms. Cats appear to be the

species giving the greatest difficulty in experimental work since the blood of young animals often contains Howell-Jolly bodies.<sup>26</sup> According to Schilling,<sup>116</sup> part of the Schmauch inner bodies of cats are Howell-Jolly bodies and part are Heinz bodies. Gross and associates<sup>23</sup> have reported that the latter are found regularly in normal animals of this species.

3 *Pre-existent theory* Two forms of this theory were advanced, the first of which was given by Schwalbe and Solley<sup>119</sup> who identified Heinz bodies with platelets. They thought that there existed within a normal red blood cell a structure similar to that of the Heinz body, the latter being liberated and appearing as a platelet in the plasma. Aside from shape, refractile properties and occurrence in the plasma, these bodies have nothing in common.

The other form of the theory was derived from the work of Schilling<sup>13 57 109-111 114</sup> who conceived of the normal erythrocyte as having a very complex structure similar to a nucleated cell. He advanced the idea that the Heinz body is a pathogenically produced form of the capsule body normally present. These capsule bodies are not observable except by special histologic and staining technic, whereas the Heinz bodies are frequently visible even in unstained preparations. This theory was also supported by the experimental investigations of Deutsch.<sup>7</sup>

Gutstein and Wallbach,<sup>36-37</sup> although agreeing in general with the pre-existent theory of Schilling, differed somewhat in details. They held to the pre-existence in normal erythrocytes of both *Innenkörper* and *Innenkörperchen*, the latter corresponding to the *Kapsulkörper* of Schilling and the former to the *Glas-körper* of Schilling. It is the *Innenkörperchen* which are the pre-existing forms of the Heinz bodies. Gutstein and Wallbach reached these conclusions also through special staining techniques which they regarded as superior to those of Schilling.

Since the special technic used to produce these structures in normal erythrocytes require somewhat severe and rough processes great artifacts may result. For this reason, Dustin<sup>17</sup> rejected this pre-existent theory.

4 *Reticulo-filamentous theory* The basophilic nature of the Heinz bodies and of the reticulocytes suggested a relationship between them. However, it is known that on supravital treatment basic stains, such as brilliant cresyl blue, precipitate material existing normally in reticulocytes in a diffuse form so that it becomes visible. On the other hand, under similar conditions the stain does not alter Heinz bodies which, if large enough, can be seen in an unstained condition. Restaining of the supravital preparation, following fixation with methyl alcohol, will color the reticulocytes but not the Heinz bodies to any marked extent, according to Dustin.<sup>17</sup>

The two kinds of bodies can be further differentiated by a study of their occurrence, Heinz bodies being found usually, though not invariably, in mature erythrocytes and rarely in the young cells (reticulocytes). Freifeld, Schilowa and Ludwinsky<sup>6</sup> have demonstrated the lack of correlation between reticulocytosis and the number of Heinz bodies present in the blood stream of poisoned animals.

5 *Detritation theory* The theory most widely held at the present time favors the view that Heinz bodies are newly formed particles generally found in mature erythrocytes and formed from them in the course of an irreversible reaction with the toxic agent or with some intermediate metabolite formed therefrom. Heub-

ner<sup>49 52</sup> and Dustin<sup>17</sup> held that these particles are derived from the erythrocytic protoplasm which suffers injury. However, investigations by Jung<sup>68 69</sup> with the electron microscope support the contention<sup>22 3</sup> that the particles are denatured proteins derived from the cell membrane. It is difficult to reconcile the latter view with the frequently observed phenomenon of Brownian movement of the Heinz particles in wet blood preparations<sup>45 121</sup>. It is quite possible that during the preparation of the blood cells for photography with the electron microscope, marked changes occur in the structure of the Heinz bodies.

Finally, it is not known for certainty where the Heinz bodies are formed. However, the fact that they can be produced *in vitro* is regarded by Moeschlin<sup>11</sup> as strong evidence of their peripheral origin.

### *Methemoglobin Formation and Heinz Bodies*

Most of the early workers assumed that there was a close connection between the formation of Heinz bodies and the formation of methemoglobin. However, in 1911 Friedstein<sup>77</sup> showed that no direct relation existed between the two. This was confirmed later by Heubner and his students<sup>4 38 46 49 50 70 78 97 117 122</sup> with many different hemolytic substances. Moeschlin<sup>88</sup> reported finding Heinz bodies in white mice treated subcutaneously or orally with sodium nitrite, although Pulina<sup>97</sup> was unable to confirm this. Following oral administration of sodium nitrite in food, Richardson<sup>99</sup> found in white Swiss mice Heinz bodies in most of the erythrocytes and also cyanosis and methemoglobinemia. On the contrary, Gutstein and Wallbach<sup>36</sup> were able to produce Heinz bodies in mice by injection of Nile blue sulfate without the formation of methemoglobin. Webster, Liljgren and Zimmer<sup>131</sup> have demonstrated in similar fashion that Heinz bodies can be produced in 75-100 per cent of the erythrocytes of mice by administration of sulfanilamide without the formation of appreciable amounts of this same blood pigment.

Goodman and Gilman<sup>37</sup> offered as a probable explanation of the action of phenylhydrazine on the erythrocyte the splitting of the hemoglobin into heme and globin, at least a portion of the remaining hemoglobin being catalyzed by the globin to form methemoglobin and perhaps other unidentified substances.

Heubner,<sup>40</sup> however, regarded the action as an opening of the tetrapyrrole ring of hemoglobin, the toxic material or some derivative thereof being able to modify the globin which was precipitated as a Heinz body and acquired an affinity for basic stains.

The presence of other blood pigment derivatives, formed in many cases along with Heinz bodies and methemoglobin, has been studied by Heubner,<sup>49 50 51</sup> by Jung,<sup>67 68</sup> and by Kiess<sup>72 73</sup> and associates. These pigments, known as verdoglobins, appear to have different spectral characteristics according to the substances producing them.<sup>72</sup> The constitution of one of the most important of these blood pigments, sulfhemoglobin (called also verdoglobin S) is unknown. Heubner and his school<sup>40</sup> believe that it contains no sulfur but this view is not widely held.<sup>39</sup> Lemberg and associates<sup>80</sup> remarked about the confused state of knowledge concerning the chemical nature of sulfhemoglobin.

Heubner<sup>50 51</sup> and Kiese and Seipelt<sup>72</sup> held that the occurrence of Heinz bodies,

methemoglobin and verdoglobin were three separate and independent phenomena, although they frequently occurred simultaneously. Kiese and Seipelt,<sup>72</sup> studying the effect of certain hemolytic substances on the blood of dogs and rats, found that Heinz bodies occurred whenever verdoglobin was present. This was explained by assuming that those substances which were toxic to the hemoglobin and converted it into verdoglobin, were also toxic to the membrane of the erythrocyte, thus denaturing it more or less completely and forming one or more Heinz bodies. Simultaneous formation of Heinz bodies and methemoglobin was explained in the same way. Heubner<sup>52</sup> attributed the nonuniform behavior of certain substances, with respect to these three phenomena, to different compounds produced during the course of intermediate metabolism of these substances.

Species differences may play an important role. For example, it is well known that it is difficult to produce methemoglobin in rabbits but not in cats,<sup>48, 81</sup> this pigment regularly occurring in the blood of normal cats.<sup>66</sup> Again, Richardson<sup>99</sup> has shown that it is difficult to produce methemoglobin in mice using sulfanilamide, sulfhemoglobin being formed much more easily. However, the reverse is true for chickens. Similar species differences have been shown by the inability of Kunz<sup>75</sup> to produce Heinz bodies in guinea pigs with m-dinitrobenzene, whereas they were readily produced by Bredow and Jung,<sup>4</sup> using cats as experimental subjects. Likewise, negative results were obtained with sulfapyridine by Moeschlin and Hurschler<sup>90</sup> when rabbits were used but mice gave positive findings. It is evident, therefore, that negative results with rabbits or guinea pigs, for example, should not be interpreted as meaning that a given substance cannot produce Heinz bodies in other species or in man. Since Heinz bodies and anemia have been shown to occur with little or no production of methemoglobin, care should therefore be taken in drawing conclusions either from the presence or absence of granules and/or altered blood pigment.

#### *Role of the Spleen in Heinz Body Phenomenon*

During the existence of Heinz bodies in experimental animals there frequently occur also secondary signs of hemolytic anemia, such as anisocytosis, polychromatophilia, and reticulocytosis. In addition, there is often evidence of splenomegaly.<sup>17</sup>

The role of the spleen in Heinz body phenomenon has been of interest ever since the primary observations of Heinz.<sup>44</sup> Hess and Muller<sup>47</sup> showed that in the macrophages of the spleen of rats poisoned by pyridine were found large numbers of Heinz body phagocytes. However, the blood leaving the spleen by the splenic vein contained no Heinz bodies and they concluded that the particles collected within the sinuses were the cause of the observed swelling. Schilling<sup>11</sup> had noted the increase in Heinz bodies of an antifebrin poisoned dog following splenectomy and Zadek and Burg<sup>128</sup> confirmed these experimental observations on several patients. Schilling<sup>11</sup> likewise observed the presence of Heinz bodies in splenectomized normal animals, i.e., those not made toxic by any Heinz body-producing material. It therefore appears as if splenectomy were a predisposing factor in the formation of Heinz bodies.<sup>16</sup>

It is difficult to reconcile this filtering action of the spleen with the experimental



observations on the persistence of Heinz bodies in the blood stream following discontinuance of the toxic material. Thus, with pyrodine, Cruz<sup>6</sup> found that the length of time required for Heinz bodies to disappear ranged from 8-9 days for rabbits to 9-18 days for dogs. Webster, Liljegren and Zimmer<sup>131</sup> observed Heinz bodies in a guinea pig for 11 days following a single exposure to stibine,  $SbH_3$ , in a rat for 33 days and in mice for 55 days after similar exposures.

Further work on the relationship between Heinz body occurrence and the action of the spleen, using various species of normal and splenectomized animals and various hemolytic substances, appears to be necessary in order to extend our knowledge of the role played by the spleen in hemolytic anemias produced by chemical agents.

### STAINING CHARACTERISTICS OF HEINZ BODIES

Enough has been mentioned to indicate that while Heinz preferred wet preparations stained with methyl violet, Ehrlich preferred fixed and stained smears. Advocates of both methods are well represented in the literature.

#### *Supravital Staining*

Friedstein,<sup>27</sup> working with Pappenheim, investigated the vital staining properties of Heinz bodies with a considerable number of basic dyes, such as brilliant cresyl blue, methyl violet, toluidine blue, azur I, malachite green, neutral red and Nile blue sulfate. The latter was regarded as being the quickest, *most intense* and easiest to use of those studied. The author found that after fixation the Heinz bodies had practically no affinity for basic stains but showed an attraction for acid stains.

Since Nile blue sulfate is but slightly soluble in water an alcoholic solution is usually used, allowing a thin film to form on a slide by evaporation. The blood is then smeared out on top of the dried film, the slide being allowed to stand in a moist chamber for 5-7 minutes before examining the cells. This is the usual Pappenheim-Schilling technic of supravital examination. A common modification of this method<sup>8</sup> consists of placing a drop of blood directly on the dried film of Nile blue sulfate and covering with a cover slip.

Gutstein and Wallbach<sup>36</sup> claimed to be able to stain both fixed and unfixed preparations by either basic or acid dyes. In their supravital staining technic, they mixed the fresh blood with aqueous solutions ( $\frac{1}{2}$ -1 per cent) of the stain and observed it between slide and cover slip.

Webster, Liljegren and Zimmer,<sup>130</sup> investigating the staining of Heinz bodies, found methyl violet and gentian violet superior to Nile blue sulfate since these violet dyes were easily soluble in water and in Locke's solution, the latter being modified so as to be more nearly isotonic with the blood to be examined.

Friedstein<sup>7</sup> confirmed Heinz's observations<sup>13</sup> that Heinz bodies could be detected in supravital preparations much earlier in the course of an intoxication than they could be detected in stained smears. Observations in this laboratory<sup>131</sup> have shown that the initial formation of Heinz bodies, when these particles are very small, can best be recognized in wet preparations, where the cells are moving and

rolling over. Since the cells are subjected to the least trauma in the wet preparations, estimation of the number of Heinz particles can be made most accurately in this way.

### *Staining of Fixed Smears*

Ehrlich's triacid stain did not prove to be very satisfactory for use in staining Heinz bodies and many other technics were devised. Panoptic staining (May-Grunwald-Giemsa) after fixation is not very satisfactory since the Heinz particles are not markedly stained. Recently,<sup>130</sup> a method has been developed utilizing the scheme of simultaneously fixing and staining the smear with a solution of methyl violet in ethyl alcohol.

Smears have the advantage of giving permanent records but the mechanical trauma during preparation of the slides frequently lead to removal of many of the Heinz bodies from the cells, as can be readily seen on inspection of the thinnest portions of the slide.

### *Heinz Bodies in Thick Drops*

Basing his work upon a technic of Ross,<sup>107</sup> Schilling<sup>112, 118, 116</sup> made extensive use of thick drop preparations, the drops after drying being hemolyzed and stained with Giemsa solution. This process has the disadvantage that the cells are largely removed so that estimation of the number of Heinz bodies in the cells is not possible. However, Schilling<sup>112</sup> regarded this method as demonstrating the presence of Heinz bodies with certainty.

### *Differential Methods*

Zadek and Burg<sup>136</sup> and Dustin<sup>17</sup> summarized the differential staining characteristics of basophilic particles, Howell-Jolly bodies, reticulocytes and Heinz bodies. The latter author cautioned also against confusion between Heinz bodies and other types of granules. Friefeld, Schilowa and Ludwinowsky<sup>38</sup> warned against confusing the marginal bodies (Randkörper) of Röhl with Heinz bodies and Jürgens and Schurer<sup>71</sup> showed how these could be distinguished. Nizer<sup>92</sup> advocated use of dark field examination as well as special staining technics for distinguishing between Heinz bodies, reticulocytes and basophilic particles. Fertman and Doan<sup>21, 22</sup> showed that Heinz bodies did not give the reactions for iron shown by siderocytes<sup>11, 34</sup> so that the two kinds of particles could be differentiated.

## EXPERIMENTAL PRODUCTION OF HEINZ BODIES

### *Production in Vitro*

Friedstein<sup>77</sup> held that Heinz bodies are formed only *in vivo*, the toxic material and the blood *in vitro* forming only methemoglobin. This was the opinion of most investigators during the first quarter of this Century. *In vitro* formation of these particles was not found by Strampelli<sup>122</sup> with pyrodine nor by Lambrechts, Nizer and Khady,<sup>6, 78</sup> with sulfonamides. The observation by Lewin<sup>4</sup> in 1889 of the formation of granules within erythrocytes by the *in vitro* action of hydroxylamine appears to have gone unnoticed. However, beginning in 1930, it was shown

by Waddell, Wolff and Lanou,<sup>18</sup> by Bratley, Burroughs, Hamilton and Kern<sup>5</sup> and by Cruz<sup>6</sup> that Heinz bodies can be produced in erythrocytes in vitro by means of acetylphenylhydrazine Moeschlin,<sup>89</sup> Nizet,<sup>92</sup> Lambrechts, Nizet and Khady<sup>76</sup> and Gajdos and Tiprez<sup>78</sup> likewise obtained positive results with phenylhydrazine. In vitro production of Heinz bodies by certain sulfonamides was reported by Moeschlin<sup>89</sup> and by Jürgens and Schürer,<sup>71</sup> although this could not be confirmed by Lambrechts and associates.<sup>78</sup> Willi<sup>122</sup> observed Heinz body formation in vitro with a preparation of guaiacol and Gross and associates<sup>92</sup> found similar action with dinitroglycol. More recently, Webster, Liljegren and Zimmer<sup>121</sup> have demonstrated this action with mouse blood for a number of hemolytic agents, using supravital staining technic.

Therefore, it can no longer be held that these morphologic changes within erythrocytes are restricted to action taking place in the living body. However, it appears that the conditions necessary for the development of Heinz bodies are much more favorable in vivo than in vitro.

#### *Production in Vivo*

Among the series of aromatic compounds which were found to produce Heinz bodies when administered to animals, the best known examples are phenylhydrazine and the acetyl derivative, pyrodine. These two drugs remained favorites, either used alone or in connection with toluenediamine, in which the acetyl compound is quite soluble, as a means of producing and studying Heinz bodies in experimental animals. However, the extremely toxic nature of these substances caused systemic effects which were undesirable.

Richardson<sup>98-100</sup> demonstrated in 1940-41 that Heinz bodies could be produced in white Swiss mice following oral administration of sulfanilamide, sulfapyridine, sulfathiazole, sulfanilylguanidine, and sodium nitrite. Using mice, positive results with nitrite were reported by Moeschlin<sup>88</sup> but this was not confirmed by Pulina.<sup>97</sup>

Renewed interest in Heinz bodies began in 1940 following the discovery by Moeschlin<sup>87</sup> of these particles in the blood of a number of persons treated with sulfapyridine. Comparison of this drug, one of the first of the sulfonamides to be used clinically, was carried out with sulfathiazole<sup>88</sup> and other derivatives.<sup>90</sup> Moeschlin and his associates found that sulfanilamide produced the greatest number of Heinz bodies and sulfathiazole the least. Since the response of rabbits to these compounds was very slight, white mice were used as experimental animals. Similar work was carried out by Hirschler<sup>61</sup> and by Lambrechts and associates.<sup>77</sup> The occasional failure to produce Heinz bodies may have been partly due to differences in modes of administration of the toxic substances and also to species differences.

Following the work of Moeschlin, Heubner and his students investigated the formation of Heinz bodies in experimental animals after the administration of a number of industrially important aromatic compounds and nitro compounds, such as nitroaniline, nitrobenzenes, nitrotoluenes, nitroglycerin and nitroglycols.<sup>4</sup>

Recently, Figge<sup>23</sup> showed that these altered erythrocytes could be easily and quickly produced in mice by the administration of sulfanilamide in their drinking water. Such a technic has been used<sup>121</sup> as a means of studying Heinz body formation for many months without the severe systemic effects produced by hydrazine derivatives.

The chemical constitution of the substances capable of producing these marked changes in erythrocytes has long been of interest. With the exceptions of chlorates and hydroxylamine, only compounds belonging to the aromatic series and containing nitrogen have been included until recently. Dustin<sup>17</sup> believes that the action by chlorates is quite different from that of other substances producing Heinz bodies. If this be accepted, it would seem that nitrogen in the form of amino or nitro groups associated with a benzene nucleus was potentially capable of inducing changes in the erythrocytes. That this is true for a variety of substituted hydrazine compounds can be seen from the work of Hueper<sup>63</sup> and of Von Oettingen and Deichmann-Gruebler.<sup>126</sup> Von Oettingen's summary<sup>125</sup> of the action of aromatic amino and nitro compounds likewise suggests the possibility of similar action with many of these industrially important substances.

In the original work by Heinz, this investigator examined the blood of the experimental animals for Heinz bodies about 24 hours following the administration of the toxic material. Recently, Gross, Bock and Hellrung,<sup>22</sup> working with cats, have shown the rapid formation of these particles. Within 10 minutes after subcutaneous injection of dinitroglycol, 100 per cent of the erythrocytes in the peripheral blood were found to contain one or more refractile particles.

Jung<sup>69</sup> has mentioned that arsine is capable of forming Heinz bodies, and Figge<sup>24</sup> has indicated that they can be formed by such varied substances as cobalt, paraminobenzoic acid and acetalilide.

Work in this laboratory<sup>131</sup> has shown that a number of other substances are also capable of inducing Heinz body formation. Among those investigated, positive results were obtained with arsine, stibine (antimony hydride), sodium nitrate and sodium nitrite besides several others used in earlier studies.

From these results it is evident that our knowledge of the relationship between chemical constitution and Heinz body formation is quite fragmentary and there exists a great need for fundamental investigations in this field.

#### *Estimation of Number of Heinz Bodies in Erythrocytes*

Since the Heinz bodies are not hemolyzed by water or saponin solution, these particles remain suspended in the solution or they can be thrown down in a centrifuge. When suspended they cause a turbidity which frequently interferes with hemoglobin determinations or counting of white cells, since in the latter case they are insoluble in acetic acid. Cruz<sup>6</sup> made use of this turbidity in quantitatively estimating the amount of Heinz bodies present. This method was also followed by Horecker<sup>19, 60</sup> and by Pimenta de Mello.<sup>98</sup> Such measurements, however, do not give the actual number of particles or the percentage of erythrocytes containing such particles. For such determinations counting may be done on fixed stained

smears<sup>120</sup> or on supravital preparations.<sup>121</sup> The latter method has the advantage that even the smaller Heinz bodies can be seen and thus the beginning stages of the phenomenon can be detected and followed.

#### CLINICAL OBSERVATIONS AND USE OF HEMOLYTIC SUBSTANCES

It should be recalled that the first observations of refractile bodies in erythrocytes were made on persons poisoned by chlorates. Ehrlich likewise observed them in certain cases of anemia and Ehrlich and Lindenthal found them in a person suffering from chronic nitrobenzene poisoning.

Following Hoppe-Seyler's work in 1885,<sup>58</sup> phenylhydrazine was extensively used as a chemical for producing experimental anemia in animals but it was not until 1918 that it was introduced by Eppinger and Kloss<sup>20</sup> for the treatment of polycythemia rubra vera. Since then it has received extensive clinical application. However, the use of this drug was attended by some danger and less toxic derivatives were sought. The acetyl compound, prepared by Liebreich,<sup>59</sup> was introduced in a somewhat impure form in England in 1887 by Dreschfeld<sup>14, 15</sup> under the name pyrodine, not for reducing the red cell count but as an antipyretic agent. Its high toxicity, which in rabbits was shown to produce jaundice and hemoglobinemia, led to its disuse until it was introduced in 1926-28 by Stone and co-workers<sup>121</sup> and by Bassett and co-workers<sup>1</sup> for the symptomatic treatment of polycythemia vera.

Little interest in Heinz bodies has been shown in this country, particularly with reference to clinical studies. This was pointed out by Cruz<sup>6</sup> and by Pimenta de Mello,<sup>96</sup> following a search of the literature on phenylhydrazine and pyrodine, and more recently by Fertman and Doan.<sup>77</sup> Almost all references to Heinz body occurrence in human blood are found in the German literature.

Schilling,<sup>112, 115</sup> who reported finding Heinz bodies in children poisoned from aniline, coined the term *Innenkörperanämien* to designate those illnesses in which the presence of inner bodies was regularly found in the erythrocytes of the circulating blood.

The discovery of Moeschlin<sup>87</sup> in 1940 that certain sulfonamides were capable of producing Heinz bodies in human beings stimulated other investigators to look for morphologic changes in the erythrocytes in their hematologic examinations. From the work of Heubner and his students, who have shown that a large number of substances are capable of producing Heinz bodies in animals, it is evident that more attention should be paid to this phenomenon in persons exposed to these substances in industry. As early as 1941, directions<sup>8</sup> were given for the microscopic examination of blood for Heinz bodies in German munition workers. In this country examination of TNT workers by Sievers et al.<sup>120</sup> indicated the need for further investigations of this phenomenon. Moreover, Gross, Bock and Hellrung<sup>23</sup> recommended that examination for Heinz bodies be made routinely in munitions plants, at least in cases of suspected poisoning, since such examinations require only a few moments and the simplest equipment. The supravital method of examination,<sup>121</sup> requiring only a drop of fresh blood, can quickly reveal even minute Heinz bodies, if present. This is in contrast to the determination of methemo-

TABLE I — *Summary of Chief Clinical Reports of Heinz Bodies in Man*

Author	Date	Diagnosis
Ricss <sup>101-103</sup>	1882-1908	Potassium chlorate poisoning
Heinz <sup>41</sup>	1890a	Pyrodiac poisoning
Ehrlich <sup>19</sup>	1892	Anemia
Ehrlich and Landeorthal <sup>18</sup>	1896	Chronic nitrobenzene poisoning
O Hnber <sup>82</sup>	1912	Potassium chlorate poisoning
Schullio <sup>112 113 115</sup>	1921 1927 1928	Chronic anisefebrio poisoning, anisef poison ing malaria blackwater fever
Zadek and Burg <sup>126</sup>	1930	Chronic myelogenous leukemia, cryptic hy- perchromic anemia, anisef poisoning
Geokin and Raschewskaja <sup>29</sup>	1933	Anisef poisoning
Freifeld Schilova and Lud vovowsky <sup>36</sup>	1937	Poisoning by anisef, dinitrobenzene and dinitrotoluene
Uogrecht <sup>104</sup>	1938	Cryogenice (phenylsemicarbazide) poisoning
Moerschlio <sup>87</sup>	1940	Sulfapyridine poisoning
Rohr <sup>105</sup>	1940	Nitrobenzene poisoning
Doenog <sup>9</sup>	1941	Dimethylenediammonodiphenylsulfone poison- ing
Dustio <sup>16</sup>	1941	Colchicine poisoning
Willi <sup>122</sup>	1942	Acetol (guanacol) poisoning
Pimcota de Mello <sup>86</sup>	1945	Polycythemia vera treated with pyrodiac
Sievers et al <sup>120</sup>	1945	Trinitrotoluene poisoning
Fertmao and Doao <sup>81 21</sup>	1945 1948	Inclusion body anemia (following use of erythrol tetramate)
Willi <sup>124</sup>	1947	Elloxan (Sulfanilamino-dimethyl pyrim- idine) poisoning

globin, which requires expensive special equipment for accurate evaluation. Moreover, indications of methemoglobin cannot be used as a criterion of the presence of Heinz bodies.

Finally, table 1 lists the chief clinical reports of Heinz bodies found in man.

## SUMMARY

Certain morphologic changes in the erythrocytes, first described accurately by Heinz in 1890, have been noted by many investigators both in experimental animals and in man. These Heinz bodies, called by various names, appear to be newly formed particles originating either from the protoplasm or the membrane of the red blood cells in the course of irreversible injury by a toxic agent. The chemical nature of these particles is uncertain but they appear to consist largely of denatured proteins. They may occur in the blood in the absence of methemoglobin or sulf hemoglobin and without anemia, these phenomena being independent of each others. Removal of these bodies from the blood stream is frequently accomplished by their destruction in the spleen, often with resulting increase in size of this organ.

From the staining characteristics of Heinz bodies it is usually possible to distinguish them from other similar particles and to measure them quantitatively. Little is known of the relationship of chemical constitution of toxic substances to Heinz body formation. The indications are that some inorganic substances are capable of inducing this action as well as many aromatic nitro and amino compounds.

The presence in the blood stream of significant amounts of Heinz bodies is evidence of some injury to the erythrocytes. If this injury is severe it may lead to marked hemolysis and anemia.

Clinical cases of Heinz body occurrence in man, due either to drugs or to industrial poisoning, are cited and the need for further work and especially for Heinz body evaluation in routine hematologic examinations is pointed out. A bibliography is included in this review of the literature covering the chief contributions to work on Heinz bodies.

## REFERENCES

- <sup>1</sup> BASSETT S. H., KILLIP T. AND McCANN W. S. Mineral exchages in man. III. Mineral metabolism during treatment of a case of polycythemia vera. *J. Clin. Investigation* 10: 771-785, 1931.
- <sup>2</sup> BIZZIZERO J. Über einen neuen Formbestandtheil des Blutes und dessen Rolle bei der Thrombose und der Blutgerinnung. *Virch. Arch. f. path. Anat.* 90: 261-332, 1882.
- <sup>3</sup> BRATLEY F. G., BURROUGHS H. H., HAMILTON D. M. AND KERN C. The effect of pyrocin poisoning on the blood and hemolytotoxic system. With special reference to the formation of Heinz Ehrlich bodies in vivo and vitro. *Am. J. M. Sc.* 182: 597-605, 1931.
- <sup>4</sup> BREHOW M. V. AND JUNG F. Studien über Methämoglobinbildung. XXV. Mitteilung. Vergleich der Giftigkeit einiger aromatischer Nitroverbindungen. *Arch. f. exper. Path. u. Pharmacol.* 100: 335-355, 1942/43.
- <sup>5</sup> CASPER J. L. Ein neues Gift. *Vierteljahrsschrift für gerichtliche und öffentliche Medicin* 16: 1-7, 1859.
- <sup>6</sup> CRUZ, W. O. Acetylphenylhydrazine anemia. I. The mechanism of erythrocyte destruction and regeneration. *Am. J. M. Sc.* 202: 781-798, 1941.
- <sup>7</sup> DEUTSCH I. Beiträge zur Genese der Ehrlich-Heinzschen Innenkörperchen. *Ztschr. f. klin. Med.* 108: 747-751, 1928.
- <sup>8</sup> DEUTSCHES REICH. Runderlass des Reichsarbeitsministers und des Reichswirtschaftsministers betr. Gesundheitsschutz der Arbeiter in der Sprengstoffindustrie. *Reichs-Gesundheitsblatt* 16: 372-377, 1941.
- <sup>9</sup> DORRINO, L. Über das Auftreten von Isonukleoproteinämien infolge Sulfonamidbehandlung. *Med. klin.* 37: 354-356, 1941.

- <sup>10</sup> VON DOMARUS, A Über Blutbildung in Milz und Leber bei experimentellen Anämien Arch f exper Path u Pharmacol 58 319-342 1908
- <sup>11</sup> DONIACH, I GRÜNEBERG, H AND PEARSON, J E G The occurrence of siderocytes in adult human blood J Path & Bact 55 23-31 1943
- <sup>12</sup> DORLANO, W A N The American Illustrated Medical Dictionary, ed 21 Philadelphia Saunders 1947
- <sup>13</sup> DOWNEY, H Editor Handbook of Hematology vol 1 New York Hoeber, 1938 Section I The erythrocyte R Isaacs
- <sup>14</sup> DRESCHFELD J Pyrocin a new antipyretic Brit M J 2 881 1888
- <sup>15</sup> — Clinical observations on pyrocin a new antipyretic Med Chron Manchester 9 89-99, 1888-89
- <sup>16</sup> DUSTIN P JR Intoxication mortelle par la colchicine Etude histologique et hématologique Bull Acad roy de méd de Belgique 6 ser, 6 505-529 1941
- <sup>17</sup> — Les corps de Heinz Leur importance en hématologie et en pathologie expérimentale Rev belge sc méd 14 48-63 1942
- <sup>18</sup> EHRLICH K AND LINDENTHAL, O Eigentümlicher Blutbefund bei einem Fall von protrahierter Nitrobenzolvergiftung Ztschr f klin Med 30 427-446 1896
- <sup>19</sup> EHRLICH, P Über schwere anämische Zustände Verhandlungen des Congresses für innere Medizin 11 33-64 1892.
- <sup>20</sup> EPPINGER H AND KLOSS K Zur Therapie der Polyzthämie Therap Monatsh 32 322-326 1918
- <sup>21</sup> FERTMAN M H AND DOAN C A Irreversible toxic inclusion body anemia, a clinical and experimental study Proc Central Soc Clin Research 18 66-67, 1945
- <sup>22</sup> — and — Irreversible toxic inclusion body anemia Blood 3 349-360 1948
- <sup>23</sup> FIOGE F H J Studies on the nature and significance of Heinz body formation in erythrocytes Anat Rec 94 461 1946
- <sup>24</sup> — (Personal communication) Quoted by Fertman M H and Doan, C A 1948 <sup>22</sup>
- <sup>25</sup> FISCHER E Ueber aromatische Hydrazinverbindungen Ber d deut chem Ges 8 589-594 1875
- <sup>26</sup> FREIFELD F SCHILOWA A AND LUDWINOWSKY, R Die Heinzsche Körperchen und die Methämoglobinbildung bei Vergiftungen mit Amido und Nitrobenzolverbindungen Folia haemat 58 333-342, 1937
- <sup>27</sup> FRIEDSTEIN DORA Experimentelle Beiträge zur Kenntnis der perniziösen Anämie und zur Pathologie der roten Blutkörperchen Folia haemat Archiv 12 239-288 1911
- <sup>28</sup> GAJDOS A, AND TIPREZ G La signification biologique des corpuscles de Heinz Compt rend Soc de biol 139 545-6 1945
- <sup>29</sup> GENKIN S AND RASCHESKAYA A Zur Klinik und Diagnostik der chronischen Anilinvergiftung Zentralbl f Gewerbhyg 10 29-36 1933
- <sup>30</sup> GLAESER K Ueber die Struktur des Erythrocyten Klin Wchnschr 12 1494-95, 1933
- <sup>31</sup> — Die Innen bzw. Kapsel (Pol)körperchen des Erythrocyten bei Pyrocinvergiftung Klin Wchnschr 13 703-705 1934
- <sup>32</sup> GOODMAN L. AND GILMAN A The Pharmacological Basis of Therapeutics New York Macmillan 1941 Pp 1149-52
- <sup>33</sup> GROSS E BOCK M AND HELLKUNO F Zur Toxikologie des Nitroglykols im Vergleich zu der des Nitroglycerins Arch f exper Path u Pharmacol 20 271-304 1942/43
- <sup>34</sup> GRÜNEBERG H Siderocytes a new kind of erythrocytes Nature London 148 114-115 1941
- <sup>35</sup> GUTSTEIN M AND WALLBACH G Über den Bau des Erythrocyten II Mitteilung Bau des Erythrocytenkörpers Virch Arch f path Anat 263 741-752, 1927
- <sup>36</sup> — AND — Über den Bau des Erythrocyten III Mitteilung Untersuchungen über die Heinz Ehrlichschen hämoglobinamischen Innenkörper Virch Arch f path Anat 267 144-154 1928
- <sup>37</sup> — AND — Über den Bau der Erythrocyten Erwiderung auf die Bemerkungen von V Schilling zu der Arbeit von Gutstein und Wallbach dieses Archiv Bd 265 Virch Arch f path Anat 267 155-157 1928
- <sup>38</sup> HAAS E Studien über Methämoglobinbildung XVIII Mitteilung Dinitroethylhydroxylamin Arch f exper Path u Pharmacol 224 130-132, 1947



- <sup>39</sup> HARRIS J S AND MICHEL, H O The formation of methemoglobin and sulfhemoglobin during sulfanilamide therapy J Clin Investigation 18 507-519 1939
- <sup>40</sup> HARTWICH, W Weiter Beiträge zur Kenntnis der Heinzschen Vergiftungskörper (Ehrlichsche hämoglobinämische Innenkörper) Folia haemat Archiv 13 257-268, 1912.
- <sup>41</sup> HEINZ, R Die praktische Verwendbarkeit von Phenylhydrazinderivaten als Fiebermittel Berl klin Wchnschr 27 47-48, 1890
- <sup>42</sup> — Morphologische Veränderungen der rothen Blutkörperchen durch Gifte Virch Arch f path Anat. 122 112-116 1890
- <sup>43</sup> — Über Blutdegeneration und Regeneration Beitr z path Anat u z allg Path 29 299-404 1901
- <sup>44</sup> — Zur Lehre von der Function der Milz Virch Arch f path Anat 168 485-500 1902.
- <sup>45</sup> — Handbuch der experimentellen Pathologie und Pharmacologie vol I part 1 Jena Fischer 1904 Pp 336-340, 396-414
- <sup>46</sup> HERKEN H Studien über Methämoglobinbildung XXVII Mitteilung Nitrierte aromatische Hydroxylamine und 3,3' Dinitroazoxybenzol Arch f exper Path u Pharmacol 202 70-78 1943
- <sup>47</sup> HEIS L AND MÜLLER H Über den Ablauf der Bluterstörung bei der Pyrodinäemie Wien klin Wchnschr 26 1831-1835, 1913
- <sup>48</sup> HEUBNER W Studien über Methämoglobinebildung XIV Arch f exper Path u Pharmacol 72 239-281 1913
- <sup>49</sup> — Methämoglobin, Innenkörper der Erythrocyten und Anämie Klin Wchnschr 26 137-141, 1941
- <sup>50</sup> — Heinzkörperchen und Blutfarbstoff Klin Wchnschr 21 520-521 1942.
- <sup>51</sup> — Reaktionsformen einiger Blutgifte Klin Wchnschr 21 961-963, 1942.
- <sup>52</sup> — Ueber Heinzkörperchen und Methämoglobinebildung Bemerkungen zu der Arbeit von Sten Moeschlin nebst Nachtrag in dieser Zeitschr 65 (1942) 345, 378 Folia haemat 67 323-324 1943
- <sup>53</sup> — AND JUNO F Zur Theorie der Chloratvergiftung Schweiz med Wchnschr 71 247-250 1941
- <sup>54</sup> — AND VON RESTORFF, H Bemerkungen zu der Arbeit von Hanny Horschler Die Toxizität der Sulfanilamid Derivate für das Blut usw Arch f exper Path u Pharmacol 197 666 1941
- <sup>55</sup> HEUER F Untersuchungen über die Zusammensetzung der Heinzkörperchen beim phenylhydrazin anämischen Tier Folia haemat Archiv 18 100-116 1914
- <sup>56</sup> HIRSCHFELD HANNA Über einige Veränderungen der roten Blutkörperchen bei experimentellen Anämien Folia haemat. Archiv 9 534-540 1910
- <sup>57</sup> —, AND HITTMAYER, A Editors Handbuch der allgemeinen Hamatologie Berlin and Wien, Urban and Schwarzenberg 1932. Book I 1st part, H. Günther
- <sup>58</sup> HOPPE SEYLER, G Über die Wirkung des Phenylhydrazins auf den Organismus Ztschr f. physiol Chem 9 34-39, 1885
- <sup>59</sup> HORECKER B L Experimental studies on the toxicity and potential dangers of trinitrotoluene (TNT) VIII Production of methemoglobin and denatured globin in dogs poisoned with TNT Pub Health Bull 285 46-49 1944
- <sup>60</sup> — The occurrence of Heinz bodies in trinitrotoluene workers J Indust Hyg & Toxicol 27 85-88, 1945
- <sup>61</sup> Huber, A Beiträge zur Giftwirkung des Dinitrobenzols Virch Arch f path Anat 126 240-270 1891
- <sup>62</sup> HUBER O Über die Blutveränderungen bei Vergiftung mit Kalium chloricum Deutsche med Wchnschr 38 1923-1924 1912
- <sup>63</sup> HUEPER W C Pathological studies on the organic effects of various hydrazine derivatives J Indust Hyg & Toxicol 18 17-36 1936
- <sup>64</sup> HORSCHLER H Die Toxizität der Sulfanilamid Derivate für das Blut und das blutbildende System im Tierversuch (Innenkörper anämien) Arch f exper Path u Pharmacol 197 438-456 1941
- <sup>65</sup> ISSEKUTZ, B VON, JR Studien über Methämoglobinebildung XVI Mitteilung Normal s Methämoglobin bei Katzen Arch f exper Path u Pharmacol 193 567-568 1939
- <sup>66</sup> ITAMI, S AND PRATT J Über Veränderungen der Resistenz und der Stomata roter Blutkörperchen bei experimentellen Anämien Biochem Ztschr 11 302-316 1909
- <sup>67</sup> JUNG, F Über das sogenannte Sulfhäemoglobin Arch f exper Path u Pharmacol 194 16-30 1939
- <sup>68</sup> — Degenerationserscheinungen an Erythrozyten Naturwissenschaften 30 472-3 1942

- <sup>10</sup> — Über Heinz Körperchen Klin Wchnschr 22 42-43 1943
- <sup>10</sup> — Studien über Methämoglobinbildung XXIX Mitteilung Chlor- und Amino-Nitrobenzols Arch f exper Path u Pharmacol 204 133-138 1947
- <sup>71</sup> JURGENS, R, AND SCHÜRER, W Über Heinsche Innenkörper und Röhlsche Randkörperchen in den Erythrozyten Schweiz med Wchnschr 75 1055-1058 1945
- <sup>72</sup> KIESE, M Die Bestimmung von Verdoglobulin im Blute Klin Wchnschr 21 565-569 1942.
- <sup>72</sup> —, AND SEIFELT L Bildung und Elimination von Verdoglobulinen Arch f exper Path u Pharmacol 200 648-683 1943
- <sup>74</sup> KUNKEL, H Chemische Beiträge zur Kenntnis der Substanz der hämoglobinämischen Innenkörper (Sog Heinzkörper) Folia haemat, Archiv 14 430-454 1912/13
- <sup>74</sup> KUNZ G Studien über Methämoglobinbildung XXII Mitteilung Vergiftung von Meerschweinchen mit m Dinitrobenzol Arch f exper Path u Pharmacol 199 508-520 1942
- <sup>76</sup> LAMBRECHTS A NIZET, A AND KHADY EL Sulfamidés, phénylhydrazine et granulations de Heinz *in vitro* Compt rend Soc de biol 140 1091-93, 1946
- <sup>77</sup> — — — — Sulfamidés et granulations de Heinz *in vivo* Compt rend Soc de biol 140 1093-94 1946
- <sup>78</sup> — — — — Sulfamidés et granulations de Heinz Experimentia 3 189-190 1947
- <sup>78</sup> LAZARUS A Die Haemoglobinaemia In Nothnagel's Spezielle Pathologie und Therapie, Book 8 Part 1, No 3 Wien Holder 1901
- <sup>80</sup> LEMBERO R HOLDEN H F LEOGE J W AND LOCKWOOD W H Studies on sulphhaemoglobin Australian J Exper Biol & M Sc 20 161-167 1942
- <sup>81</sup> LEVIN S J Shoe-dye poisoning Relation to methamoglobin formation J A M. A. 89 2178-2180, 1927
- <sup>82</sup> LEWIN L Über Hydroxylamin Ein Beitrag zur Kenntnis der Blutgifte Arch f exper Path Pharmacol 25 306-325, 1889
- <sup>82</sup> LIEBREICH, O Bericht über das Pyrodin Therap Monatsh 2 557-558 1888
- <sup>81</sup> MARCHAND, F Ueber die Intoxication durch chloresaurer Salze Virchows Arch f path Anat 77 455-488 1879
- <sup>81</sup> — Über die giftige Wirkung der chloresaurer Salze Arch f exper Path u Pharmacol 22 201-232, 1887, 23 273-315 347-392, 1887
- <sup>84</sup> MARKOFF, N G Über Veränderungen des Blutes im Verlauf der Therapie mit Sulfanilamid und seinen Derivaten Schweiz med Wchnschr 73 656-662 1943
- <sup>87</sup> MOESCHLIN, S Innenkörper Anämien durch Entstehung von Methämoglobin infolge Dageñan Wirkung (Sulfapyridin) Schweiz med Wchnschr 70 786-790 1940
- <sup>88</sup> — Versuche über die Abhängigkeit der Toxizität verschiedener Sulfanilamidderivate auf die Erythrozyten (Innenkörperanämien) von ihrer chemischen Struktur Schweiz med Wchnschr 71 789-792 1941
- <sup>88</sup> — Versuche über die Entstehung von Innenkörpern in Erythrocyten (Heinzsche Körperchen) *in vivo* und *in vitro* durch Sulfanilamidderivate und Phenylhydrazin Folia haemat 65 345-381 1941
- <sup>89</sup> — AND HIRSCHLER H Vergleichende Untersuchungen über die verschiedene Toxizität des Dageñans (Sulfapyridin) und des Ciba 3714 (Sulfathiazol) auf das rote Blutbild (Innenkörperanämien durch Methämoglobinbildung) Schweiz med Wchnschr 70 972-975 1940
- <sup>91</sup> MORAWITZ P AND PRATT J Einige Beobachtungen bei experimentellen Anämien München med Wchnschr 55 1817-1819 1908
- <sup>91</sup> NIZET A Recherches sur la physiopathologie des hématies I Nouvelles techniques pour l'étude et la distinction des hématies granulées Acta med Scandinav 17 199-215, 1944
- <sup>91</sup> — L'action de la phénylhydrazine sur les hématies *in vitro* Compt rend Soc de biol 140 1094-97 1946
- <sup>91</sup> PATENHEIM A Über die Vitalfärbung und die Natur der vitalfärbbaren Substanzen der Blutkörperchen Folia haemat Archiv 1 289-301 1911
- <sup>91</sup> — — — — AND SCHULKE T Weitere Mitteilungen zur Kenntnis der Heinzschen Vergiftungs Körperchen Folia haemat Archiv 13 205-214 1912
- <sup>91</sup> PEREIRA DE MELLO R Formação de grânulos de Heinz em doente de policitemia vera submetido a tratamento pela 2 etil hidrazina Rev brasil de med 1 387-399 1945

- <sup>97</sup> PULINA B Studien über Methämoglobinbildung XXIV Mitteilung Wirkungen von Salpeter und Salpetersäureestern auf das Blut Arch f exper Path u Pharmacol 200 324-334 1942-43
- <sup>98</sup> RICHARDSON A P Effects of continued administration of sulfanilamide on the blood J Pharmacol & Exper Therap 70 370-377, 1940
- <sup>99</sup> — Effect of sulfur compounds in the diet on sulfanilamide cyanosis and anemia J Pharmacol & Exper Therap 71 203-209 1941
- <sup>100</sup> — Comparative effects of sulfonamide compounds as to anemia and cyanosis J Pharmacol & Exper Therap 72 99-111 1941
- <sup>101</sup> RIESS L Über Vergiftung mit chloresurem Kalium Berl klin Wchnschr 19 785-788 1882.
- <sup>102</sup> — Beitrag zur Lehre von der Wirkung der chlorsuren Salze auf den thierischen Organismus Zentralbl f Physiol 1 213-220, 1887-8
- <sup>103</sup> — Über Vergiftung mit chloresurem Kalium oebst Bemerkungen über Hamoglobin Ausscheidung in den Nieren Arch f exper Path u Pharmacol, Supplbd 1908 460-468
- <sup>104</sup> RÖHL Über acute und chron Intoxicationin durch Nitrokörper der Benzolreihe Dissertation Rostock 1890 Quoted by Hnber A <sup>61</sup>
- <sup>105</sup> ROHR, K Das menschliche Knochenmark Leipzig Thieme 1940 P 162.
- <sup>106</sup> ROSENTHAL, F Die sogenannte Pachydermie der Erythrocyten bei Phenylhydrazinämie Folia haemat, Archiv 10 253-276, 1910
- <sup>107</sup> ROSS, R The thick film process for the detection of organisms in the blood Thompson Yates and Johnston Lab Rep Liverpool 5 117-119 1903
- <sup>108</sup> SÄTTLER J Über experimentell erzeugte allgemeine Resistenzerböhung der roten Blutkörperchen Folia haemat Archiv 9 216-232, 1910
- <sup>109</sup> SCHILLING V Der Säugetiererythrocyt als vollständige Zelle und seine Beziehung zum Blutplättchen München med Wchnschr 58 445-449 1921
- <sup>110</sup> — Arbeiten über Erythrocyten Arbeit II bis VII Membran Glaskörper Kapselkörper Blutplättchen und Kernreste, protoplasmatische Grundstruktur und vollständiger Erythrocyt Folia haemat Archiv 14 95-248 1922.
- <sup>111</sup> — Die Zelltheorie des Erythrocyten als Grundlage der klinischen Wertung anämischer Blutbefunde Virch Arch f path Anat 234 548-601 1921
- <sup>112</sup> — Erweiterte hämatologische Verwertung des dicken Bluttröpfens für Kernkugeln Innenkörper Endothelien u a Deutsche med Wchnschr 47 825-826 1921
- <sup>113</sup> — Fall von autochthoner Innenkörperanämie Klin Wchnschr 6 1493 1927
- <sup>114</sup> — Über den Bau der Erythrocyten Bemerkungen zu d.r Arbeit von Gutstein und Wallbach dieses Archiv Bd 263 H 3 Virch Arch f path Anat 265 536-540, 1927
- <sup>115</sup> — Über einen als Herzfehler erscheinenden Fall von Innenkörperanämie aufgedeckt als chronische Antifebrilvergiftung und Coffeinsucht Ztschr f klin Med 108 709-736 1928
- <sup>116</sup> — Das Blutbild und seine klinische Verwertung eds 9 and 10 Jena Fischer, 1933 Pp 105-106 422.
- <sup>117</sup> SCHLIMME H Studien über Methämoglobin XXVI Mitteilung Phenyl und Tolyldihydroxylamin Arch f exper Path u Pharmacol 202 60-69 1943
- <sup>118</sup> SCHMAUCH, G Über endoglobuläre Körperchen in den Erythrocyten d.r Katz. Virch Arch f path Anat 156 201-244 1899
- <sup>119</sup> SCHWALBE E, AND SOLLEY J B Die morphologischen Veränderungen d.r Blutkörperchen speziell der Erythrocyten bei der Toluylendiamin Vergiftung Virch Arch f path Anat 168 399-431 1922.
- <sup>120</sup> SIEVERS, R F LAWTON A H ET AL. A medical study of the effect of TNT on workers in a bomb and shell loading plant Pub Health Bull 291, 48-50 1945
- <sup>121</sup> STONE, C T HARRIS, T H, AND BODANSKY M The treatment of polycythemia vera Report of two cases J A M A 101 495-498 1933
- <sup>122</sup> STRAMPPELLI B Sul significato dei corpi di Heinz Ehrlich e sull azione esercitata dell apparato macrofagico sulla ematopoiesi Haematologica 11 397-419 1930
- <sup>123</sup> SUZUKI T Weitere Beiträge zur Kenntnis der Erythrocytenveränderungen bei Pyrodivergiftung Folia haemat Archiv 13 225-256 1912.
- <sup>124</sup> UNORICHT M. Die Wechselbeziehungen der menschlichen Reticulocyten im Knochenmark und im peripheren Blut Folia haemat. 60 145-204, 1938
- <sup>125</sup> VON OETTINORN W F The aromatic amino and nitro compounds, their toxicity and potential dangers Washington, 1941 Pub Health Bul No 271

- 126 —, AND DEICHMANN-GRUEBLER W On the relation between the chemical constitution and pharmacological action of phenylhydrazine derivatives *J Indust Hyg & Toxicol* 18 1-16 1936
- 127 —, NEAL, P A STEVENS R F ET AL Xylidine (C,C-dimethylaniline) Its toxicity and potential dangers as compared with those of aniline and an appraisal of the potential hazards from its use in blending gasoline *Nat Inst Health Bull* 188 33 37-40 1947
- 128 WAOOELL, WOLFF, AND LANOU The mechanism of pyridin anemia Quoted by Bratley Burroughs, Hamilton and Kern <sup>2</sup>
- 129 WARBURG O, KUBOWITZ F AND CHRISTIAN W Über die Wirkung von Phenylhydrazin und Phenylhydroxylamin auf den Stoffwechsel der roten Blutzellen (Methode zur Messung des Stoffwechsels roter Blutzellen) *Biochem Ztschr* 242 170-205 1931
- 130 WEBSTER, S H. LILJEGREN E J, AND ZIMMER D J Rapid staining of Heinz bodies in smears *Stain Technol* 23 97-98 1948
- 131 — AND — Heinz body formation by certain chemical agents *J Pharmacol & Exper Therap* 95 201-211 1949
- 132 WILHELM, H Studien über Methämoglobiobildung XXIII Mitteilung Blutwirkung aliphatischer Salpetersäureester *Arch f exper Path u Pharmacol* 200 305-323 1942/43
- 133 WILLI H Methaemoglobinbildung und Innenkörperanämie durch Anastil *Ann paediat* 159 282-83 1942.
- 134 — Innenkörperbildung durch Elkosin und spontane Innenkörperbildung *Schweiz med Wchnschr* 77 243 1947
- 135 WINOGRADOW B Zur Frage der Kali chloricum Vergiftung *Virch Arch f patb Anat* 190 92-124, 1907
- 136 ZADEK, I AND BURD K. Innenkörperanämien *Folia haemat* 41 333-355, 1930

# STUDIES ON THE DESTRUCTION OF RED BLOOD CELLS

## V IRREVERSIBLY SICKLED ERYTHROCYTES THEIR EXPERIMENTAL PRODUCTION IN VITRO

By SHU CHU SHEN, M D , ELEANOR M FLEMING, A B AND W B CASTLE, M D

IT IS well known that in fresh preparations of the blood of patients with sickle cell anemia the erythrocytes can be sickled immediately by displacement of the oxygen from the hemoglobin, for example, by carbon dioxide or by nitrogen. These sickled cells can be no less rapidly restored to their normal form when the blood is reexposed to oxygen. However, even in stained smears of the peripheral blood of some of these patients a few sickled cells may be present, despite the inevitable exposure of the film of fresh blood to atmospheric oxygen.<sup>1-3</sup> These cells, then, differ from the majority of the sickled erythrocytes artificially produced in fresh preparations in that they have somehow acquired an inability to revert to the normal discoidal form upon exposure to oxygen. Moreover, though crescentic or elliptic, these cells do not display filamentous extremities as do freshly sickled erythrocytes in wet preparations.<sup>2, 4-6</sup>

Although in patients with sickle cell disease the majority of the erythrocytes when exposed to a range of hypotonic concentrations of sodium chloride exhibit a so-called increased osmotic resistance, critical study of the phenomenon indicates that in certain patients a small percentage of the erythrocytes may actually possess a slightly decreased osmotic resistance relative to the normal range, that is, they are hemolyzed in concentrations of sodium chloride, somewhat more concentrated than those which initiate the osmotic lysis of normal blood. Because it is possible to cause any type of red cell so far studied to acquire decreased resistance to osmotic lysis by sterile incubation in vitro,<sup>7</sup> the question arose as to whether they irreversibly sickled erythrocytes and those erythrocytes with decreased resistance to osmotic lysis are in fact the same cells. It also appeared to be possible that both characteristics were the result of the same process, namely, stagnation of the red cells in vivo in the tissue capillaries.<sup>3, 7, 8</sup> Accordingly, the peripheral bloods of 4 patients with sickle cell disease were studied with respect to the natural presence of irreversibly sickled erythrocytes and as to their artificial production in vitro.

### METHODS

The conventional characteristics of the formed elements of the peripheral blood were determined by the usual methods. The percentages of irreversibly sickled erythrocytes in samples of capillary or of defibrinated venous blood following exposure to air or to 90 per cent oxygen and 10 per cent carbon dioxide in a tonometer were determined while counting 1,000 or more red cells in blood films prepared

From the Thorndike Memorial Laboratory Second and Fourth Medical Services (Harvard) Boston City Hospital and the Department of Medicine Harvard Medical School Boston Mass  
This investigation was aided in part by a grant from the John and Mary R. Markle Foundation

and treated with Wright's stain in the usual manner. The percentages of reticulocytes were also determined in blood spread upon coverslips previously prepared with a dried film of brilliant cresyl blue. Thereafter the dried blood films were counterstained with Wright's stain and the number of reticulocytes determined by counting 1,000 or more red blood cells.

In order to observe the effect of sterile incubation at 37.5°C. for 24 hours *in vitro* on the reversibility of the sickling phenomenon, 7 cc. samples of sterile defibrinated blood were equilibrated in 250 cc. tonometers with gas mixtures containing either 90 per cent oxygen and 10 per cent carbon dioxide or 90 per cent nitrogen and 10 per cent carbon dioxide. Each tonometer, which was equipped with a long glass capillary pipet inserted through a hole in a rubber stopper closing the open end, was sterilized prior to each experiment. With the tonometer lying on its side on a table with the stopcock open, the apparatus was carefully rotated back and forth about its long axis usually during three periods of 3 minutes each, separated by short intervals. During each of these periods the gas mixture, which was stored in a cylinder equipped with a reducing valve, after bubbling through water, was allowed to flow freely through appropriate rubber tube connections to the capillary pipet and so into and through the tonometer. The stopcock and the inflow tubing were then closed. During a 24-hour period in an air incubator the blood in the tonometers was reequilibrated three or four times with the same gas mixture. At the end of the incubation period the blood in the tonometers was again carefully equilibrated with whichever of the two gas mixtures was required by the experimental procedure, in the same fashion as in the beginning.

Whenever a sample of blood was to be removed for study, the tonometer was held in a vertical position. The long glass capillary pipet, which then dipped beneath the surface of the blood, was operated as a bulb pipet and a small amount of blood was sucked into it and so removed from the tonometer without contact with room air. After withdrawal from the tonometer, the tip of the pipet was inserted beneath the surface of a sterile pool, on the surface of a glass slide, composed of two drops of a 40 per cent formalin solution diluted 10 times by volume with physiologic salt solution.<sup>9</sup> A drop of blood was then expelled and immediately mixed with the formalin solution. From this mixture as well as directly from other drops of blood expelled from the pipet without contact of its tip with coverslips, blood smears were prepared using either plain coverslips or coverslips previously filmed with brilliant cresyl blue. If the experiment was to continue, the remaining blood was expelled from the pipet which was then reinserted in the tonometer with appropriate sterile precautions. The blood remaining in the tonometer was then equilibrated twice with the appropriate gas mixture as described above and the apparatus was returned to the incubator. Later the blood films were treated with Wright's stain in the usual manner and the percentages of sickled erythrocytes and of sickled reticulocytes were determined.

## RESULTS

As shown in table 1, sickled red cells persisted in the blood of 3 of the 4 patients, Cases 2, 3, and 4, after exposure of capillary or defibrinated venous blood to atmos-

phoric air or even after the further equilibration of the latter with a gas mixture consisting of 90 per cent oxygen and 10 per cent carbon dioxide. In Cases 3 and 4, the number of sickled erythrocytes differed strikingly at the time of the two observations made on each patient's blood. As noted by others,<sup>2, 4-8</sup> these irreversibly sickled cells, although exhibiting the sickle or oat shaped form in fixed as well as in wet preparations, did not possess the filaments which are seen in wet preparations of blood artificially sickled by exposure to nitrogen or to carbon dioxide gas.

TABLE 1.—Percentages of Irreversibly Sickled Erythrocytes in Peripheral Blood of 4 Patients with Sickleemia

Case Number	Capillary Blood	Venous blood	
		De fibrinated in air	De fibrinated and oxygenated
1	0	0	0
2	2	1	1
3	3	—	—
3 (after splenectomy)	21.5	16.8	10.8
	4.2	5.0	5.0
4	14.0	12.9	11.8
	4.6	5.0	5.6

TABLE 2.—Characteristics of the Peripheral Blood of 4 Patients with Sickleemia

Case Number	R B C Mills	Hgb. %	W B C Thous	Reticles %	Hematocrit %	MCV $\mu^3$	MCHC %	MCH $\gamma$	H I units	Osmotic fragility of R.B.C.				
										Hemolysis per cent				
										1	5	10	50	75
										NaCl per cent				
1	5.35	81	26.2	7.4	38.9	72.7	32.4	23.6	10+	0.58	0.34	0.29	0.20	0.16
2	2.72	52	8.0	2.2	23.9	87.9	33.9	29.8	5+	0.38	0.31	0.28	0.21	0.16
3	3.07	57	13.1	7.6	27.5	89.6	32.0	28.7	10	0.44	0.38	0.36	0.27	0.23
4	2.69	46	12.2	9.0	22.4	79.6	33.2	26.4	7	0.47	0.34	0.26	0.18	0.14
Normal										0.43	0.40	0.39	0.36	0.33

\* 15.6 grams of hemoglobin are considered to be 100 per cent.

In table 2 are shown the peripheral blood values including the data on quantitative osmotic fragility studies on the blood of the 4 patients. It will be noted that in the blood of Case 1 a portion of the red cells were more susceptible than are normal cells to lysis by hypotonic salt solution although no irreversibly sickled erythrocytes were seen in the blood films from this patient. Thus in Case 1, 1 per cent of the red cells were hemolyzed in 0.58 per cent NaCl instead of, as in the normal individual, in 0.43 per cent NaCl. In Cases 2, 3, and 4, on the other hand, 1 per cent of the red cells were hemolyzed in 0.38, 0.44, and 0.47 per cent NaCl respectively, and thus like the rest of the red cells of all of the patients, were less susceptible than are normal red cells to lysis by hypotonic solutions.

In an attempt to modify the capacity of the red cells for change in shape, sickling

of the erythrocytes was prevented as far as possible during sterile incubation of blood samples for 24 hours in equilibrium with a gas phase containing 90 per cent oxygen and 10 per cent carbon dioxide. As shown in table 3, experiment C, these erythrocytes almost completely lost their capacity to become sickled upon subsequent reduction of the hemoglobin by exposure to 90 per cent nitrogen and 10 per cent carbon dioxide. Likewise, except in Case 3 where the effect was only partial, the erythrocytes which were kept sickled in the nitrogen-carbon dioxide atmosphere during the incubation period largely lost their ability to reassume the dis-

TABLE 3—Percentages of Sickled Erythrocytes in Blood Samples from 4 Patients with Sickleemia following Incubation for 24 Hours in the Presence and Absence of Oxygen Respectively

Exp	Characteristics of blood samples	Percentage of sickled erythrocytes					
		Case 1	Case 2	Case 3		Case 4	
		Non retics	Non retics	Non retics	Retics	Non retics	Retics *
A	Defibrinated venous blood immediately after equilibration with 90 per cent O <sub>2</sub> and 10 per cent CO <sub>2</sub>	0	1	5	3	5 6	2
B	Defibrinated venous blood after incubation in 90 per cent O <sub>2</sub> and 10 per cent CO <sub>2</sub> for 24 hours	0	1	11 0 (10)	4 6	7 0 (11)	2 0
C	Same after final reequilibration in 90 per cent N <sub>2</sub> and 10 per cent CO <sub>2</sub>	0	2	13 5 (15)	5 0	6 0 (14)	0
D	Defibrinated venous blood immediately after equilibration with 90 per cent N <sub>2</sub> and 10 per cent CO <sub>2</sub>	(70)	(88)	(30)	—	(25)	—
E	Defibrinated venous blood after incubation in 90 per cent N and 10 per cent CO for 24 hours	90	88	44 (35)	40	25 (26 9)	60
F	Same after final reequilibration to 90 per cent O and 10 per cent CO	75	84	38 (27)	32	28 6 (24)	22

\* Figures in column are percentages of sickled reticulocytes in terms of total reticulocytes

( ) Figures in parentheses are the results of observations using the formalin technic.

coidal form upon exposure to the oxygen-carbon dioxide gas mixture (experiment F). In table 3 are also shown the immediate effects of equilibration with the oxygen-carbon dioxide gas mixture (experiment A) and with the nitrogen-carbon dioxide gas mixture (experiment D). Also included are the results of the various experimental procedures upon the reticulated erythrocytes of Cases 3 and 4. The prolonged incubation in the oxygen-carbon dioxide gas mixture appeared effectively to prevent the subsequent sickling of the reticulocytes upon exposure to a nitrogen-carbon dioxide gas mixture (compare experiment C with experiment D).



However, incubation in the nitrogen-carbon dioxide gas mixture was not as effective in rendering reticulocytes irreversibly sickled when subsequently exposed to oxygen as it was in the case of the nonreticulated erythrocytes (compare experiment E with experiment F). The figures obtained with the formalin technic are shown in parentheses in table 3. The percentages of sickled erythrocytes in the smears prepared from the formalin solution agreed reasonably well with those made in the usual fashion. The technic could not be employed for reticulocytes, which were rendered unable to take the brilliant cresyl blue stain by previous exposure to formalin.

### DISCUSSION

The fact that the sickling of the erythrocytes strikingly increases the viscosity<sup>7</sup> of the blood provides an obvious explanation of the characteristic pathologic lesions of sickle cell disease, namely, the congestion of the capillaries and the multiple thromboses and infarcts including frequently the total atrophy of the spleen.<sup>2, 7, 8</sup> However, many sickled red cells apparently traverse these areas of lowered oxygen tension and thus with proper precautions against exposure to air are demonstrable in wet preparations of venous blood. The readiness with which these cells revert to the discoidal form distinguishes them from the irreversibly sickled erythrocytes which may also be present in such wet preparations and which form the subject of this communication. Only the irreversibly sickled forms, however, are observed in the usual stained blood films. The hypothesis examined experimentally here is that the persistently sickled form has been assumed because of prolonged or repeated intermittent exposure to anoxia and consequent erythrocytosis in the capillaries of various organs.<sup>2, 7, 8</sup> Janet Watson<sup>6</sup> infers the effectiveness of stagnation *in vivo* from the finding of Diggs and Bibb of irreversibly sickled forms in the pleural or ascitic fluids of patients whose peripheral blood showed none of these forms. Clearly, from the experiments reported here, the effect of sterile incubation *in vitro* upon erythrocytes maintained in the sickled form under anoxic conditions is to cause loss of ability to revert to the discoidal form upon reexposure to oxygen. Moreover, these incubated sickled erythrocytes resemble the irreversibly sickled forms seen in fixed blood smears with respect to their lack of the hairlike processes frequently extending from the ends of the crescents that are characteristic of the sickled forms artificially produced in wet preparations by exposure to nitrogen-carbon dioxide gas mixtures.<sup>2, 4-6</sup>

Although irreversibly sickled reticulocytes are rarely seen in the peripheral blood of patients with sickle cell disease,<sup>1-3, 6</sup> these cells are capable of sickling *in vitro* if their hemoglobin is sufficiently reduced.<sup>1, 2, 6</sup> The present experiments indicate that after incubation in the nitrogen-carbon dioxide gas mixture a larger proportion of the reticulated than of the nonreticulated erythrocytes, especially in the blood of Case 4, were able to revert to the discoidal form upon re-exposure to oxygen. This finding confirms the suggestion already made by others<sup>3, 6</sup> that the acquisition of a permanently sickled form requires that the red cell receive a sufficiently long or repeated exposure to whatever bodily processes are concerned in order to allow for maturation of the reticulocyte to the more adult form. However,

although the blood of Case 1 showed an increased osmotic fragility of a small proportion of the red cells, a finding which conceivably could be due to erythro-stasis *in vivo*,<sup>7</sup> no irreversibly sickled red cells were present. The fact that irreversibly sickled reticulocytes are so rarely seen in the peripheral blood is strong evidence against the possibility that they are young cells recently delivered by the bone marrow.

It may be argued that exposure to atmospheric oxygen in the preparation of the blood films would invalidate the experiments in which formalin fixation was not used. However, reversal of sickling due to exposure to atmospheric oxygen would be expected only in the case of films made from blood removed from the tonometer after equilibration with the nitrogen-carbon dioxide gas mixture, as, for example, in experiments C, D, and E of table 3. The differences between the numbers of sickled red cells in the ordinary smears and in the formalin fixed smears do not significantly alter the conclusions drawn from these experiments.

### CONCLUSIONS

1 The peripheral blood of patients with sicklemia when examined in wet preparations without contact with air may contain two distinct types of sickled erythrocytes.

The first type, which exhibits filamentous processes extending from the ends of the crescentic forms, resembles those produced by exposure of the blood to nitrogen or to carbon dioxide gas *in vitro*. Exposure to oxygen causes these red cells to revert to the discoidal form.

The second type appears as sickle or oat shaped forms without filaments, in fixed as well as in wet preparations of the blood, and does not revert to the discoidal form upon exposure to oxygen. Such red cells rarely exhibit the vital staining properties of reticulocytes. In the blood of one patient their presence did not correlate with the presence of red cells of relatively increased osmotic fragility.

2 When samples of the blood of 4 patients with sicklemia were incubated for 24 hours at 37.5°C in the absence of oxygen, the nonreticulated red cells largely lost their ability to resume the discoidal form when exposed again to oxygen. To a considerable extent, however, reticulocytes retained their ability to revert to the discoidal form. Similar incubation in the presence of oxygen caused loss of the ability of both adult and reticulated red cells to sickle when subsequently deprived of oxygen.

3 These studies confirm the hypothesis that sufficient intermittent or continuous stagnation of the red cells in various organs *in vivo* with consequent sickling may result in the production of irreversibly sickled forms.

4 The fact that reticulocytes do not as readily acquire the property of becoming irreversibly sickled after incubation *in vitro* as do nonreticulated red cells may explain the fact that irreversibly sickled reticulocytes are rarely seen in stained blood films.

### REFERENCES

- 1 SYDENSTRICKER, V. P., MILLER, W. A. AND HOUNEAL, R. W. Sickle cell anemia: Report of two cases in children with neoplasms in one case. *Am. J. Dis. Child.* 6: 13-19-3.

- <sup>2</sup> DODS, L. W., AND BIBB, J. The erythrocyte in sickle cell anemia, *J. A. M. A.* 112, 695, 1939
- <sup>3</sup> MURPHY, R. C. AND SHAPIRO, S. The pathology of sickle cell disease. *Ann. Int. Med.* 23, 376, 1945
- <sup>4</sup> GRAHAM, G. S. AND McCARTY, S. H. Sickle cell (menisocytic) anemia. *South. M. J.* 23, 598, 1930.
- <sup>5</sup> SYDENSTRICKER, V. P. Sickle cell anemia. *M. Clin. North America* 12, 1451, 1919
- <sup>6</sup> WATSON, J. A study of sickling of young erythrocytes in sickle cell anemia. *Blood* 3, 465, 1948
- <sup>7</sup> HAM, T. H., AND CASTLE, W. B. Relation of increased hypotonic fragility and of erythrocytosis to the mechanism of hemolysis in certain anemias. *Tr. A. Am. Physicians* 55, 127, 1940
- <sup>8</sup> BAUER, J. Sickle cell disease. Pathogenic, clinical and therapeutic considerations. *Arch. Surg.* 41, 1344, 1940
- <sup>9</sup> SHERMAN, I. J. The sickling phenomenon with special reference to the differentiation of sickle cell anemia from the sickle cell trait. *Bull. Johns Hopkins Hosp.* 67, 309, 1940

## OVALOCYTOSIS ASSOCIATED WITH THE SICKLE CELL TRAIT

By ROBERT S FADEM, M D

THE PATIENT described in this report is a young Negro male whose blood showed both ovalocytosis and sickling. Only one other instance of the coexistence of ovalocytosis and the sickling phenomenon has been found in the literature.<sup>8</sup> In that instance some of the ovalocytic cells became sickled in fresh sealed blood preparations. In the patient reported here it was found that only the discoid red blood cells were capable of sickling, the ovalocytes did not undergo this change.

Ovalocytosis has been reported in both sexes and in both white and Negro races.<sup>11-13</sup> The precursors of the oval shaped cells show no detectable morphologic abnormality, and the anomaly apparently first makes its appearance in the reticulocyte stage.<sup>17</sup> The factors which determine the unusual shape of these cells have not been established.

It has been suggested that ovalocytosis is hereditary and is transmitted as a simple mendelian dominant<sup>6,7</sup> Entire families with the trait have been studied.<sup>1,2,4,12</sup> It has also been suggested that (a) these cells represent a structural adaptation to some unknown constitutional factor<sup>10</sup>, (b) that they have an intraerythrocytic susceptibility to some extraerythrocytic influence, either one of which, or both, may be congenital.<sup>7</sup>

Several different observations have been made upon ovalocytes in fresh sealed preparations. One case has been described in which the ovalocytes became more round in appearance when sealed for long periods of time in fresh preparations.<sup>12</sup> Other cases have been reported in which the ovalocytes did not change shape in such preparations.<sup>3, 5</sup> One case has been reported in which some of the ovalocytic cells became sickled in such preparations.<sup>8</sup>

We wish to report in this paper a patient observed during routine hematologic study who presented the peripheral blood picture of ovalocytosis associated with the sickle cell trait. Sickling was observed to occur only in the normal appearing discoid red blood cells.

## CASE REPORT

(Hospital File # 345974) W J 25 year old Negro male entered the San Diego Naval Hospital with the complaints of intermittent chest pain bone aches and weakness of approximately three months duration. He had been seen by his private physician on three occasions prior to his admission to the hospital. At each visit the patient was told he had an anemia (because of the unusual appearance of his red blood cells). Treatment with iron and multiple vitamins was instituted at each visit. As the patient was a government employee he was admitted to the San Diego Naval Hospital for a thorough examination on July 21 1947.

The physical examination was entirely normal. On routine hematologic studies his peripheral blood was observed to contain 76 per cent ovalocytes. Because of this observation further studies were made that revealed the data shown in tables 1, 2, and 3.

Fresh wet preparations under cover glass sealed with vaselin<sup>®</sup> showed sickling of the normal appearing discoid red cells within 18 hours. The ovalocytes did not change their shape after 72 hours in the same preparations (see fig. 3).

Manuscript prepared while author was a medical officer in the United States Navy

## OVALOCYTOSIS AND THE SICKLE CELL TRAIT

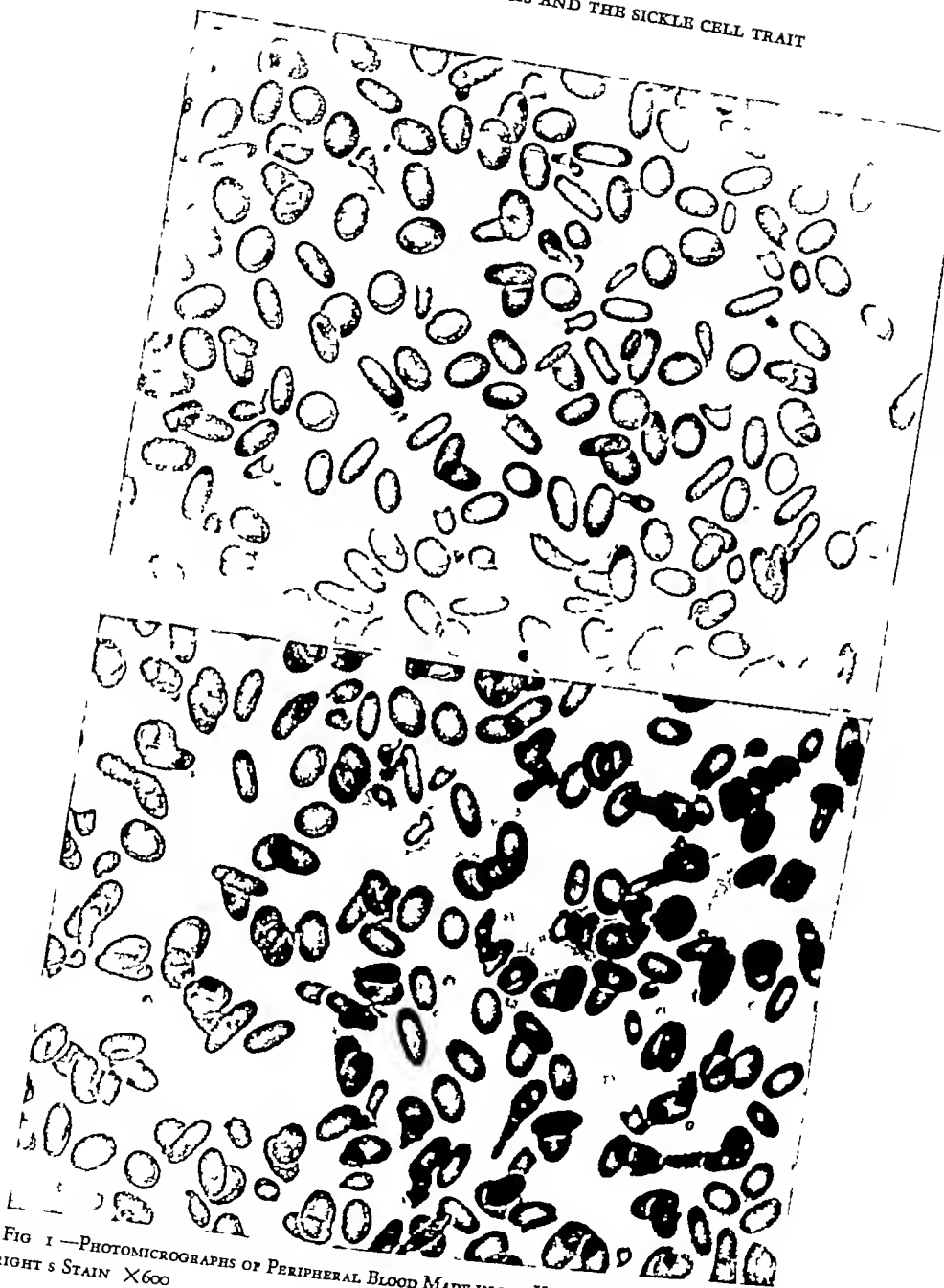


FIG 1 —PHOTOMICROGRAPHS OF PERIPHERAL BLOOD MADE IN THE USUAL MANNER AND STAINED WITH WRIGHT'S STAIN  $\times 600$

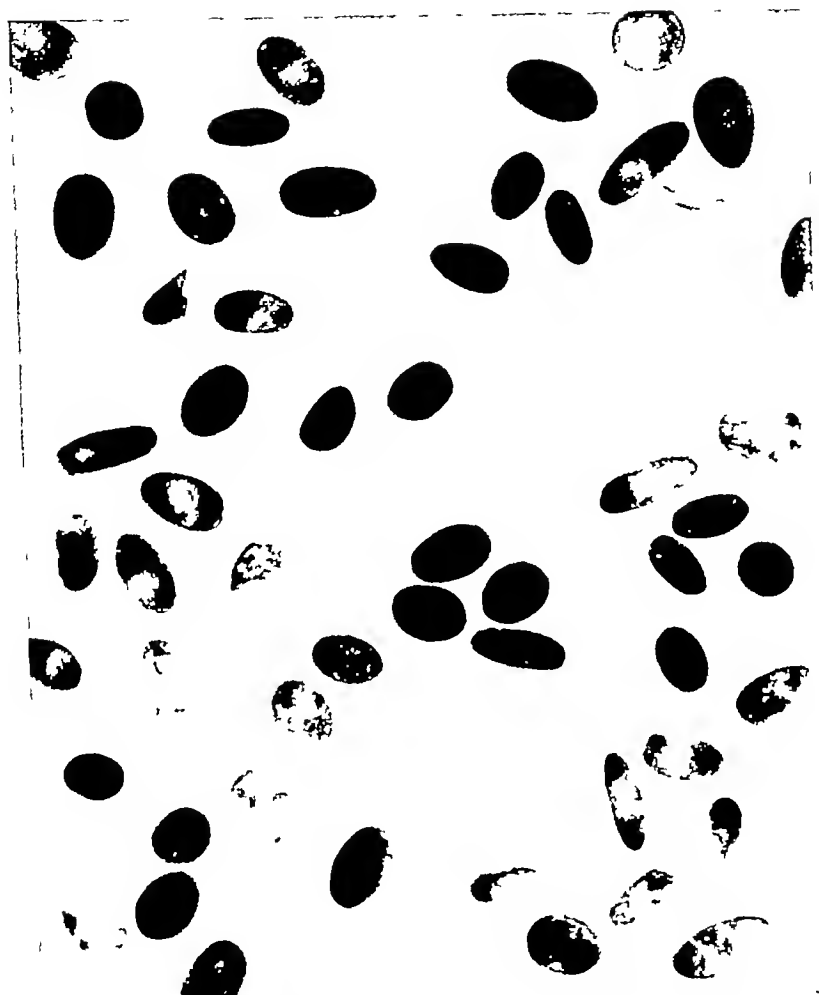


FIG. 2.—REPRODUCTION OF A PHOTOMICROGRAPH OF CELLS AS SEEN AFTER IMMEDIATE FIXATION OF PERIPHERAL BLOOD WITHDRAWN UNDER OIL.  $\times 950$

Bone marrow examination revealed a normal bone marrow. The red blood cell precursors were all of normal shape. There was no evidence of hyperplasia of the red blood cell elements.

The patient's symptoms disappeared after he was told he had no serious blood disease. He was discharged on the fifth hospital day completely asymptomatic. He was seen again six months after discharge on January 20, 1948, at which time blood studies were essentially unchanged from admission studies.

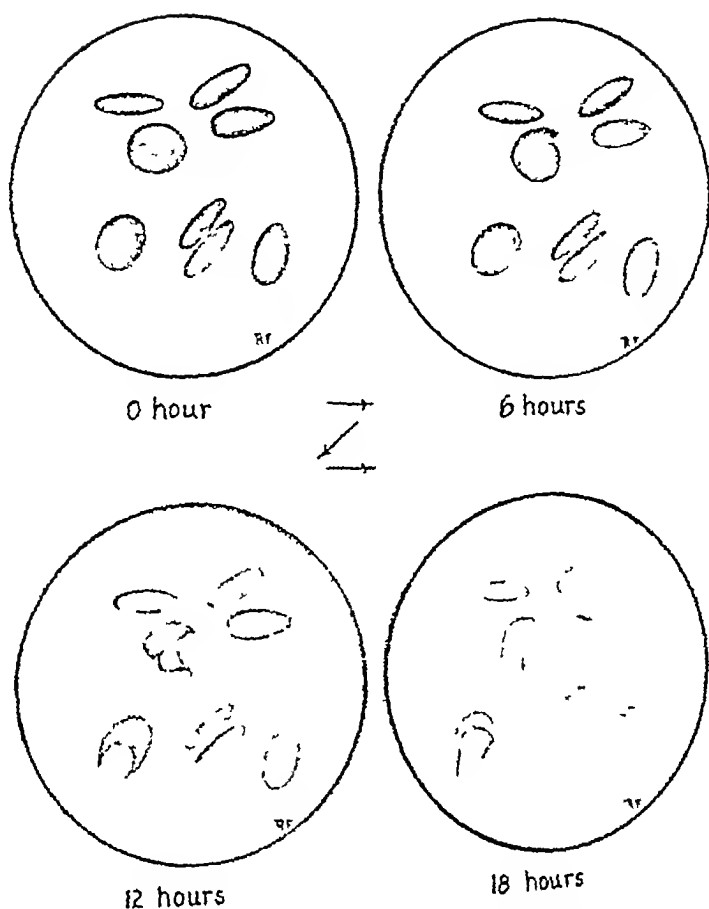


FIG. 3—FRESH FIXED PREPARATION SHOWING SICKLING OF NORMAL APPEARING DISCOID CELLS. Drawings made at 0, 6, 12 and 18 hours. Ovalocytes showed no sickling.

#### SUMMARY

1. A patient has been presented whose circulating red blood cells were composed of 65-84 per cent ovalocytes, 3-11 per cent sickled cells, and some normal appearing discoid cells.
2. The red blood cell counts and the blood indices were within normal limitations.
3. The red blood cells showed an increased resistance to hypotonic saline solutions.

TABLE 1

Date	WBC	RBC	Hb	PCV	MCV	MCHb*	MCHb Conc *
	<i>per cu mm</i>	<i>per cu mm</i>	<i>Gms per 100 cc</i>	<i>%</i>	<i>cu <math>\mu</math></i>	<i>micro micrograms</i>	<i>%</i>
8-22-47	7,500	4.7	14	41	87	30	34
8-25-47	8,200	4.9	14.5	42	86	30	34
8-27-48	7,250	4.9	14.5	42	86	30	34

\* figured to the nearest whole number

TABLE 2

Date	Fresh Fixed Preparation*		Fresh Sealed Preparation at 72 hours	
	Ovalocytes	Sickled Cells	Ovalocytes	Sickled Cells
	<i>%</i>	<i>%</i>	<i>%</i>	<i>%</i>
8-22-47	76	8	75	16
8-25-47	84	3	84	14
8-27-47	65	11	67	21

\* To determine the exact percentages of ovalocytes and sickled cells circulating in the peripheral blood at any one time blood was withdrawn from a vein into a syringe under oil directly into a ten per cent formalin in saline fixing solution. The fixed cells were then placed on glass slides, the fixing solution allowed to evaporate and the cells stained with Wright's stain<sup>8</sup>

TABLE 3

Date	Fragility Tests		
	Subject	Hemolysis began	Hemolysis completed
8-22-47	Control	40	32
	Patient	32	28
8-27-47	Control	42	36
	Patient	38	30

4 The peripheral blood showed a daily variation in the percentage of circulating ovalocytes, from 65 per cent to 84 per cent, and in the percentage of circulating sickle cells, from 3 per cent to 11 per cent

5 After 72 hours in fresh wet preparations the per cent of ovalocytes remained essentially unchanged from that of fresh fixed blood

6 The percentage of sickled cells was found to be increased after 18, 24, and 72 hours in fresh wet preparations as compared to the percentage of sickled cells found in fresh fixed preparations

7 Some of the normal appearing discoid red blood cells were observed to sickle in fresh wet preparations within 18 hours

#### CONCLUSION

We have shown that this patient's red blood cells demonstrated the condition referred to as ovalocytosis. In addition, the patient's normal appearing discoid



red blood cells demonstrated the sickling phenomenon when observed in fresh sealed wet preparations. Therefore, we wish to present this patient as one whose peripheral blood showed the result of two different abnormalities one, ovalocytosis, and the other, the sickle cell trait.

## REFERENCES

- <sup>1</sup> BISHOP, F. W. Elliptical human erythrocytes. *Arch. Int. Med.* 14: 388, 1914.
- <sup>2</sup> CHENET, G. Elliptic human erythrocytes. *J. A.M.A.* 98: 878, 1932.
- <sup>3</sup> FLORMAN, A. L. AND WINTROBE, M. M. Human elliptical red corpuscles. *Bull. Johns Hopkins Hosp.* 63: 209, 1938.
- <sup>4</sup> HELZ, M. K., AND MENTEN, M. L. Elliptocytosis, a report of two cases. *J. Lab. & Clin. Med.* 29: 185, 1944.
- <sup>5</sup> HUNTER, W. C., AND ADAMS, R. B. Hematologic study of three generations of a white family showing elliptical erythrocytes. *Ann. Int. Med.* 2: 1162, 1928.
- <sup>6</sup> ———. A further study of a white family showing elliptical erythrocytes. *Ann. Int. Med.* 6: 775, 1933.
- <sup>7</sup> MILLER, J. H., AND LUCAS, M. A. Elliptical erythrocytes in human blood. *Amer. J. Clin. Path.* 8: 391, 1938.
- <sup>8</sup> POLLACK, L. H., AND DAMESHEK, W. Elongation of the red blood cells in a Jewish family. *Am. J. M. Sc.* 138: 822, 1934.
- <sup>9</sup> SHERMAN, I. J. *The sickling phenomenon, with special reference to the differentiation of sickle cell anemia from the sickle cell trait.* *Bull. Johns Hopkins Hosp.* 67: 309, 1940.
- <sup>10</sup> TERRY, M. O., HOLLINGSWORTH, E. W., AND EUGENIO, V. Elliptical human erythrocytes. *Arch. Path.* 23: 193, 1932.
- <sup>11</sup> WAGNER, F. An intermediate type of ovalocytosis in a Negro. *J. Lab. and Clin. Med.* 30: 454, 1945.
- <sup>12</sup> WINTROBE, M. M. *Clinical Hematology.* Philadelphia, Lea & Febiger, 1946.
- <sup>13</sup> WYANDT, H., BANCROFT, P. M., AND WINSHIP, T. O. Elliptic erythrocytes in man. *Arch. Int. Med.* 66: 1043, 1941.

# THE BLOOD AND BONE MARROW IN PATIENTS WITH CIRRHOSIS OF THE LIVER

By LAWRENCE BERMAN, M D , ARNOLD R. AXELROD, M D , THOMAS N. HORAN, M D , SAMUEL D. JACOBSON, M D , ELWOOD A. SHARP, M D , AND ELMORE C. VONDERHEIDE, M D

## INTRODUCTION

A RELATIONSHIP between hematologic changes and cirrhosis of the liver has been shown experimentally<sup>25 27 28</sup> and clinically<sup>71 81 94</sup>. Many other-wise excellent studies are not completely satisfactory because the diagnoses of hepatic cirrhosis have not been verified by biopsy. The advantages of biopsy in the diagnosis of hepatic diseases have been emphasized and illustrated by Hofbauer, Evans and Watson,<sup>39</sup> and others.<sup>19 39 49 56 28-28</sup>

## CASES AND METHODS

This study is based on a review of the literature and analysis of 25 cases with diagnoses of hepatic cirrhosis verified by biopsy of the liver. Complete blood studies with simultaneous aspiration biopsy of sternal marrow obtained within one-half to twenty-four hours before the liver specimen was removed were carried out on all patients. There were 19 males, and 6 females. The age range of the patients was 31 to 71 years, 76 per cent ranged between 43 and 68 years. The case histories, clinical and pathologic diagnoses are given in the appended case reports.

Peripheral blood studies, including determinations of volumetric data and corpuscular constants were carried out according to methods described by Wintrobe.<sup>22</sup> Normal ranges for erythrocyte, leukocyte and platelet counts, mean corpuscular volume, and mean corpuscular hemoglobin referred to are those stated by Wintrobe and found to be in agreement with observations made in our laboratory. Differential counts of cells in the bone marrow were based on enumeration of a minimum of 2000 cells. Serum protein levels were determined by the method described by Osgood.<sup>64</sup>

The hematologic terminology used by us follows that in current use by Downey<sup>24</sup> and Jones.<sup>44</sup>

Liver specimens were obtained peritoneoscopically. The evaluation of the liver lesion was based chiefly on the degree of fibrosis and atrophy according to the following schedule: Grade I cirrhosis, traces of fibrosis; Grade II cirrhosis, definite fibrosis plus atrophy of hepatic cells; Grade III cirrhosis, fibrous tissue equal to hepatic parenchyma; Grade IV cirrhosis, fibrous tissue exceeds hepatic parenchyma. In addition, other factors such as fatty metamorphosis, lymphocytic infiltration, and atypical bile duct or hepatic cell regeneration were taken into consideration.

From the Departments of Pathology and Medicine, Wayne University College of Medicine, and the City of Detroit Receiving Hospital. Technical assistance was furnished by the Anemia Laboratory, Outpatient Department, Harper Hospital.

The degree of hemosiderosis was determined by examination of sections stained for iron

The sternal marrow was obtained and processed by the methods described by two of us (L B and A R A) <sup>8</sup> Estimations of megakaryocyte content of marrow samples were based on counts of megakaryocytes in serial sections of aspirated marrow particles Fifty consecutive fields outlined by the Whipple eye piece disc were examined at 100 micron intervals The total numbers of megakaryocytes in the fifty fields were compared with similar counts on material from 10 healthy individuals

All studies were made shortly after admission of the patients to the hospital, before various therapeutic measures were undertaken

## THE BLOOD PICTURE IN PATIENTS WITH CIRRHOSIS OF THE LIVER

### Literature

*Anemia* The common occurrence of anemia in patients with cirrhosis of the liver has been mentioned frequently <sup>1 2 13 14 27 32 33</sup> The presence of anemia is not considered to be dependent on bleeding <sup>2 27-28 46</sup> It has been stated that the anemia is usually either macrocytic or normocytic, it is not hypochromic unless hemorrhage is a complicating factor <sup>31</sup> Wintrobe<sup>32</sup> noted that while macrocytic anemia is found in cases of liver disease of various types, and is most common in cirrhosis, the anemia is present only in instances of disease of long duration and wide extent. The reported frequencies of macrocytosis, macrocytic or hyperchromic anemia vary greatly For example Benhamou<sup>4</sup> observed macrocytic anemia in only 11 per cent of 35 patients, whereas Rosenberg and Walters<sup>71</sup> found that macrocytosis, almost invariably associated with anemia, was present in 89.7 per cent of 48 patients Others<sup>1 15 33</sup> have reported incidences from 43 to 91 per cent. Some of the inconsistency in the reports may be due to inadequacy of determining cell size without the use of data obtained with the hematocrit

The anemia of cirrhosis may also be normochromic or normocytic.<sup>8</sup> In a group of patients reported by Fellinger and Klima <sup>27 32</sup> 5 per cent of 40 patients had normochromic anemia Anderson and Strandell<sup>2</sup> reported normochromic anemia in 48 per cent of 61 patients who did not have bleeding from the gastrointestinal tract Although bleeding is not an essential factor in the pathogenesis of hyperchromic or normochromic anemia in patients with hepatic cirrhosis, it is usually a prominent cause for microcytic hypochromic anemia <sup>1 2 4 27 32</sup>

A significant number of patients do not have appreciable anemia When bleeding is carefully excluded as has been done in the two reports by Fellinger and Klima <sup>27 32</sup> 34 per cent of patients in various stages of the disease are free of anemia The dictum that anemia in cirrhosis is proportional to the duration and extent of the disease as is true in experimental animals,<sup>37</sup> does not seem to be substantiated by our review of the literature

*Qualitative Changes in Erythrocytes* With regard to qualitative changes in erythrocytes in the anemic patients the literature presents conflicting views According to Whitby and Britton<sup>41</sup> there is a generalized macrocytosis without gross anisocytosis or poikilocytosis Alessandrini et al.<sup>1</sup> described a characteristic isomacrocytosis while others<sup>4 26</sup> emphasized the prominence of increased anisocytosis We have observed increased anisocytosis and poikilocytosis in some of our patients but these features were neither present nor absent with sufficient regularity to be of value for distinguishing between the macrocytic anemia of cirrhosis and that of other conditions

*Leukocytes* There are few detailed reports of the leukocytic picture in patients with cirrhosis of the liver Some authors<sup>1 24 46 73 76 93</sup> were unable to find characteristic changes in the leukocytes Others<sup>29 32 46</sup> have discussed the occurrence of leukopenia especially in uncomplicated cases <sup>27 33</sup> It is generally recognized that some patients exhibit a normal leukocyte count while others experience leukocytosis after paracentesis, surgical operations or infections Masio<sup>42</sup> felt that the appearance of coarse azurophilic granules in increased numbers in the majority of monocytes is a pathognomonic sign of cirrhosis of the liver Saragea and Seicaresco<sup>75 76</sup> stated that even in leukopenic cases there is a high

percentage of neutrophils. The latter statement implies that lymphopenia may be partly or largely responsible for the leukopenias.

**Platelets.** Occasional references to the platelet counts<sup>1 28 40 46 74</sup> include few with particular emphasis on platelets. The counts were subnormal in a varying proportion of cases. Monges, Poinso and Fructus<sup>59</sup> made a special study of platelets in 15 patients. In 12 there was thrombocytopenia of moderate degree. Morlock and Hall<sup>61</sup> found thrombocytopenia in 17.5 per cent of 80 patients. A hemorrhagic tendency was present in many of their patients regardless of the presence or absence of low platelet counts, but it was relatively twice as frequent when thrombocytopenia was associated.

### *Results of the Present Study*

**Anemia.** Our cases were analyzed for the presence or absence of anemia, and the characteristics of the anemia, when present. Twenty-one patients (84 per cent) had anemia. Sixteen of the anemic patients exhibited macrocytosis, 3 had normocytosis, and 2 had microcytosis. Approximately three-fourths of our patients had macrocytic anemia or macrocytosis on their initial studies. In 84 per cent of the cases with macrocytic anemia the mean corpuscular hemoglobin values were normal or elevated. Microcytic hypochromic anemia was associated with chronic and acute blood loss from the gastrointestinal tract in 1 of 2 patients with this type of anemia.

TABLE 1—Erythrocyte counts and grade of cirrhosis in 25 patients

Grade of cirrhosis	Number of cases	Range RBC (per cu mm)	Average RBC (per cu mm)
1	5	2,450,000-4,020,000	3,530,000
2	9	2,750,000-5,000,000	4,068,000
3	4	2,790,000-3,870,000	3,393,000
4	7	2,590,000-4,950,000	3,700,600

There was no correlation between the severity of the anemia and the grade of cirrhosis (table 1).

**Leukocytes.** The total leukocyte counts ranged between 1,960 and 47,200 per cubic millimeter. Five patients had counts below 5,000, 10 had counts within the normal range (5,000 to 10,000), and 10 had leukocytosis. We did not observe a relationship between the total leukocyte counts and the presence or absence of ascites or bleeding, since leukopenia, leukocytosis and normal counts were present in both these categories. However, leukocytosis occurred with greater frequency among patients with fever or infection, or both. In other words, the presence of cirrhosis did not inhibit the leukocytic response to infection or toxemia. The severity of the liver lesion was not a factor affecting the total leukocyte counts.

In sixteen of the 25 patients (64 per cent) there was absolute lymphopenia (less than 1,500 lymphocytes per cubic millimeter). This was present regardless of whether the total counts were depressed, normal, or elevated (table 2). In fact, several of the lowest absolute counts of lymphocytes occurred in patients with leukocytosis. With the exception of 2 cases, one of which was that of a patient with both cirrhosis and chronic lymphatic leukemia, the absolute counts of lymphocytes were at low normal or lower than normal levels. Absolute lymphopenia was the most constant significant alteration in the leukocytic picture in patients with hepatic cirrhosis.

A special study was made of the peripheral blood smears in an effort to discover morphologic changes in the leukocytes of our patients. Except for occasional instances in which toxic changes of neutrophils and monocytes were seen, there were no significant alterations of the morphology of the cells.

TABLE 2.—Total leukocyte counts, absolute counts of lymphocytes and types of leukocyte picture in 25 patients with cirrhosis of the liver

Case	Total leukocytes (per cu mm)	Lymphocytes (per cu mm)	Remarks	
1	5,500	1,710	Neutropenia	
2	5,300	1,060		Lymphopenia
3	11,000	880	Neutrophilia	Lymphopenia
4	12,500	2,500	Neutrophilia	
5	6,650	1,930		
6	6,500	910		Lymphopenia
7	8,500	1,360	Neutrophilia	Lymphopenia
8	7,650	1,960		
9	3,350	670	Neutropenia	Lymphopenia
10	4,950	540		Lymphopenia
11	10,450	1,150	Neutrophilia	Lymphopenia
12	11,350	680	Neutrophilia	Lymphopenia
13	7,550	1,660		
14	23,300	930	Neutrophilia	Lymphopenia
15	4,850	580		Lymphopenia
16	10,500	1,360	Neutrophilia	Lymphopenia
17	24,500	980	Neutrophilia	Lymphopenia
18	10,800	2,710	Neutrophilia	
19	8,000	2,320		
20	12,050	3,380	Neutrophilia	
21	13,950	1,120	Neutrophilia	Lymphopenia
22	47,200	9,920	Neutrophilia	Lymphocytosis*
23	3,800	990	Neutropenia	Lymphopenia
24	5,900	1,480		Lymphopenia
25	3,200	930	Neutropenia,	Lymphopenia

\* Case 22 complicated by chronic lymphatic leukemia.

TABLE 3.—The platelet counts in 25 patients with cirrhosis of the liver

Platelets (per cu mm)	No. of cases
Less than 50,000	2
50,000 to 100,000	3
100,000 to 150,000	8
150,000 to 300,000	11
300,000 to 400,000	1

**Platelets** The platelet counts observed in our patients are shown in table 3. The counts were determined indirectly by comparing the number of erythrocytes with that of the platelets in stained blood smears. In our hands, the method yields

normal values ranging from 150,000 to 350,000 per cubic millimeter. The counts were in the low normal range or significantly lowered in 13 of 25 patients.

### *Discussion of the Peripheral Blood Findings*

We may summarize the available information regarding the peripheral blood findings in patients with cirrhosis of the liver as follows:

1. Anemia, usually of macrocytic or normocytic type, is of common occurrence.
2. In the majority of instances of macrocytosis, the mean corpuscular hemoglobin values are normal or elevated.
3. Bleeding is not an essential factor in the production of macrocytic or normocytic anemia in cirrhosis.
4. Microcytic hypochromic anemia is suggestive of chronic bleeding when it occurs in patients with cirrhosis of the liver.
5. The severity of anemia is not proportional to the duration or extent of the liver lesion, although this appears to be true of experimental cirrhosis of the liver in rats.
6. There are no constant significant qualitative changes in the erythrocytes or leukocytes.

7. In uncomplicated cases, leukopenia is likely, but the presence of cirrhosis does not prevent the leukocytic response to infection or other complications.

8. Lymphopenia, regardless of the total leukocyte count, is the most constant significant change in the leukocytic picture in patients with cirrhosis. This point seems to have been overlooked. In Masina's data on 20 patients with the disease the total leukocyte counts ranged from 3,200 to 8,360 per cubic millimeter.<sup>55</sup> The absolute counts of lymphocytes were less than 1,500 in 17 of his cases (85 per cent). Russo<sup>74</sup> supplied data on 14 patients with total leukocyte counts from 3,000 to 16,000. In ten cases (71 per cent) the lymphocytes were below 1,500 per cubic millimeter and, in an additional case, the lymphocyte count was 1,564.

9. The platelet count is in the low normal range or significantly lowered in the majority of patients. Those with severe liver damage had lower counts, on the average, than was the case for patients with slight liver damage in our material. Hemorrhagic phenomena are approximately twice as frequent in patients with thrombocytopenia as compared with patients having normal platelet levels.

The pathogenesis of anemia in cirrhosis of the liver is obscure. While it is clear that microcytic hypochromic anemia can be attributed to chronic blood loss in nearly all instances, our material indicates that normocytic or macrocytic anemias are not dependent on bleeding, as has also been shown by others.<sup>2, 27-29</sup> "It has been shown that some patients with cirrhosis of the liver have greatly increased plasma volumes."<sup>28</sup> The importance of hemodilution as a factor resulting in depressed erythrocyte levels needs further evaluation.

Various theories concerning the cause of macrocytosis have been offered. Among them are (1) Defective storage or metabolism of the anti-pernicious anemia principle (Wintrobe,<sup>22</sup> Wintrobe and Shumacker<sup>24</sup>), (2) Increased incidence of reticulocytes (which are larger than mature cells) (Rosenberg and Walters<sup>71</sup>), (3) Swelling of erythrocytes as a result of direct action of retained bile derivatives in

the peripheral blood (Meulengracht<sup>57</sup>) None of these theories is satisfactory for the following reasons (1) The hematopoietic factor has been demonstrated in the livers of patients dying of extensive hepatic disease,<sup>77</sup> (2) Marked macrocytosis may be present without marked reticulocytosis (see our cases 5, 6 and 12), (3) The degree of icterus is not proportional to the degree of macrocytosis and, as Boros<sup>18</sup> pointed out, not only is the cell volume increased but also the hemoglobin content and color index is elevated, which would not be the case if a plasma factor had caused simple swelling of the erythrocytes

It cannot be denied, however, that increased hemolysis may play a role This view is based on the observations of Watson<sup>80</sup> who cited 8 patients with macrocytic hemolytic anemia and cirrhosis of the liver, and those of others<sup>1</sup> who have utilized quantitative determinations of urobilinogen excretion in addition to complete blood studies

It is of interest that both lymphopenia and hyperglobulinemia occurred in the majority of our patients This combination recalls the work of Dougherty and White<sup>82</sup> who have demonstrated the relationship between pituitary adrenal cortical secretion on the one hand, and lymphopenia and hyperglobulinemia on the other It has also been shown that many kinds of stimuli can cause the adrenal cortical activation which results in this combination of phenomena Among them are administration of large doses of estrogens In view of the work of Glass, Edmonson and Soll<sup>82</sup> which reveals increased excretion of free estrogens by patients with cirrhosis of the liver as a result of failure of the damaged liver to inactivate estrogens, the possibility exists that the lymphopenia and hyperglobulinemia so often seen in patients with cirrhosis, in part at least, have their origin in adrenal cortical activation

## THE BONE MARROW PICTURE IN PATIENTS WITH CIRRHOSIS OF THE LIVER

### *Literature*

Reports of bone marrow can be divided into two groups those based on autopsy material and those based on aspiration of marrow from the living patient

*Autopsy Material* In most instances the marrow was studied by means of gross inspection, or by examination of sections prepared from various bones<sup>12 14 22 24-28 30 32 33 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62 64 66 68 70 72 74 76 78 80 82 84 86 88 90 92 94 96 98 100 102 104 106 108 110 112 114 116 118 120 122 124 126 128 130 132 134 136 138 140 142 144 146 148 150 152 154 156 158 160 162 164 166 168 170 172 174 176 178 180 182 184 186 188 190 192 194 196 198 200 202 204 206 208 210 212 214 216 218 220 222 224 226 228 230 232 234 236 238 240 242 244 246 248 250 252 254 256 258 260 262 264 266 268 270 272 274 276 278 280 282 284 286 288 290 292 294 296 298 300 302 304 306 308 310 312 314 316 318 320 322 324 326 328 330 332 334 336 338 340 342 344 346 348 350 352 354 356 358 360 362 364 366 368 370 372 374 376 378 380 382 384 386 388 390 392 394 396 398 400 402 404 406 408 410 412 414 416 418 420 422 424 426 428 430 432 434 436 438 440 442 444 446 448 450 452 454 456 458 460 462 464 466 468 470 472 474 476 478 480 482 484 486 488 490 492 494 496 498 500 502 504 506 508 510 512 514 516 518 520 522 524 526 528 530 532 534 536 538 540 542 544 546 548 550 552 554 556 558 560 562 564 566 568 570 572 574 576 578 580 582 584 586 588 590 592 594 596 598 600 602 604 606 608 610 612 614 616 618 620 622 624 626 628 630 632 634 636 638 640 642 644 646 648 650 652 654 656 658 660 662 664 666 668 670 672 674 676 678 680 682 684 686 688 690 692 694 696 698 700 702 704 706 708 710 712 714 716 718 720 722 724 726 728 730 732 734 736 738 740 742 744 746 748 750 752 754 756 758 760 762 764 766 768 770 772 774 776 778 780 782 784 786 788 790 792 794 796 798 800 802 804 806 808 810 812 814 816 818 820 822 824 826 828 830 832 834 836 838 840 842 844 846 848 850 852 854 856 858 860 862 864 866 868 870 872 874 876 878 880 882 884 886 888 890 892 894 896 898 900 902 904 906 908 910 912 914 916 918 920 922 924 926 928 930 932 934 936 938 940 942 944 946 948 950 952 954 956 958 960 962 964 966 968 970 972 974 976 978 980 982 984 986 988 990 992 994 996 998 1000</sup> The obvious advantage of autopsy material is that the diagnoses of hepatic cirrhosis can be accepted as verified On the other hand, sectioned material obtained after death is less suitable for differentiation of marrow cells than is the case for marrow in the form of freshly prepared imprints or smears Autolytic changes which develop rapidly after death preclude accurate identification of many of the cells of the marrow<sup>70</sup> However, the distribution of cellular marrow can be observed and in fresh tissue, it is possible to distinguish the main varieties of leukocytoid and erythroid cells

Various authors have described extension of hematopoietic marrow into the shafts of the femurs,<sup>14 22 24-28 30 32 33 34 36 38 40 42 44 46 48 50 52 54 56 58 60 62 64 66 68 70 72 74 76 78 80 82 84 86 88 90 92 94 96 98 100 102 104 106 108 110 112 114 116 118 120 122 124 126 128 130 132 134 136 138 140 142 144 146 148 150 152 154 156 158 160 162 164 166 168 170 172 174 176 178 180 182 184 186 188 190 192 194 196 198 200 202 204 206 208 210 212 214 216 218 220 222 224 226 228 230 232 234 236 238 240 242 244 246 248 250 252 254 256 258 260 262 264 266 268 270 272 274 276 278 280 282 284 286 288 290 292 294 296 298 300 302 304 306 308 310 312 314 316 318 320 322 324 326 328 330 332 334 336 338 340 342 344 346 348 350 352 354 356 358 360 362 364 366 368 370 372 374 376 378 380 382 384 386 388 390 392 394 396 398 400 402 404 406 408 410 412 414 416 418 420 422 424 426 428 430 432 434 436 438 440 442 444 446 448 450 452 454 456 458 460 462 464 466 468 470 472 474 476 478 480 482 484 486 488 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In general, it has been stated that erythroblastic hyperplasia occurs in both the normal sites of hematopoiesis and in the sites representing replacement of yellow marrow by hemopoietically active marrow There are, however, a few reports of lymphocytic myelocytic, myeloblastic or fatty marrow<sup>34 42 52 54 56 58 60 62 64 66 68 70 72 74 76 78 80 82 84 86 88 90 92 94 96 98 100 102 104 106 108 110 112 114 116 118 120 122 124 126 128 130 132 134 136 138 140 142 144 146 148 150 152 154 156 158 160 162 164 166 168 170 172 174 176 178 180 182 184 186 188 190 192 194 196 198 200 202 204 206 208 210 212 214 216 218 220 222 224 226 228 230 232 234 236 238 240 242 244 246 248 250 252 254 256 258 260 262 264 266 268 270 272 274 276 278 280 282 284 286 288 290 292 294 296 298 300 302 304 306 308 310 312 314 316 318 320 322 324 326 328 330 332 334 336 338 340 342 344 346 348 350 352 354 356 358 360 362 364 366 368 370 372 374 376 378 380 382 384 386 388 390 392 394 396 398 400 402 404 406 408 410 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912 914 916 918 920 922 924 926 928 930 932 934 936 938 940 942 944 946 948 950 952 954 956 958 960 962 964 966 968 970 972 974 976 978 980 982 984 986 988 990 992 994 996 998 1000</sup> Rossier<sup>72</sup> who described an occasional myeloblastic reaction in addition to the usual erythropoietic reaction in his autopsy series, called attention to the fact that autopsy material is not satisfactory for identification of cells and that it was probable that the so-called myeloblasts represented erythropoietic elements Fellingner and Alima<sup>77</sup>

studied a group of 48 patients with Laennec's cirrhosis. In each case occult and gross bleeding from the gastrointestinal tract had been carefully excluded. In the autopsied cases they found red marrow in the shafts of the femurs even in the absence of bleeding.

**Biopsy Material.** The advantages of aspiration biopsy of the sternum are that the bone marrow can be examined during life before autolytic changes have occurred, the time of aspiration can be selected to coincide with the liver biopsy, the identification of cells can be made with relative ease and in addition that material for sectioning is obtainable for use in estimating the cellularity, fat content, and frequency of certain irregularly distributed cell types such as megakaryocytes.<sup>8, 8, 9</sup> In nearly all instances, investigations utilizing biopsy material have been based on the technic of sternal aspiration. The experimental work of Stasney and Higgins<sup>82</sup> was based on fresh autopsy material from rats. Since these authors prepared dry films in addition to sections of bone marrow, their observations can be compared with those of others using similar preparations from patients.

Schulten<sup>79</sup> was of the opinion that there are marrow changes in all cases of hepatic cirrhosis. Although Isaacs<sup>41</sup> described both hypocellular and hypercellular marrows in 8 patients, the preponderance of reports implies a regular appearance of cellular or hypercellular bone marrow during life.<sup>1, 4, 8, 29, 47, 50, 81, 84</sup>

**Fat Content of Marrow.** The relative fat content of aspirated marrow in cirrhosis has not been studied sufficiently. Klima<sup>47</sup> stated that although the cellularity of the sternal marrow is markedly increased, the fat content may be considerable. In one patient in whom two simultaneous sternal aspirations were done, Pizzolato and Stasney<sup>84</sup> found relative fat volumes of 1 and 3 per cent.

**Cell Content of Marrow.** The average myeloid-erythroid volume (ME volume) that is the average relative volume occupied by nucleated cells in aspirated centrifuged sternal marrow in 20 patients with hepatic cirrhosis was found to be 13 per cent by Limarzi et al.<sup>81</sup> This was considered about twice the normal value. In an additional case reported by others<sup>88</sup> the ME volumes were 5 and 10 per cent in marrow from two different sites in the sternum.

**Myeloid-erythroid Ratios.** A number of reports<sup>1, 8, 29, 51, 68-71, 81, 82</sup> include data concerning the myeloid-erythroid ratios (ME ratios) which represent the relative frequencies of myeloid leukocytes and erythroblasts<sup>8</sup> in the aspirated specimens of marrow. In some cases we have calculated the ratios from the authors' data. The combined statistics from a total of 39 cases yield ME ratios ranging from less than 1:1 to 6:1. The ME ratio was 1:1 or less in 14 cases, 2:1 to 3:1 in 23 cases, and over 4:1 in 2 cases. Benhamon and Nouch<sup>18</sup> mentioned a reversal of the ME ratio so that erythroblasts predominated, as was also observed by Limarzi and co-workers.<sup>81</sup> A similar reversal of the ME ratio indicating relative increase of erythropoiesis has been noted in experimental cirrhosis of the liver in rats.<sup>82</sup> The opinion that erythroblastic hyperplasia occurs frequently in patients with hepatic cirrhosis is upheld by additional observations on biopsy material.<sup>1, 3, 4, 29, 31, 36, 47, 50, 63, 68, 81</sup> The presence of erythroblastic hyperplasia is not dependent on bleeding.<sup>79</sup>

**Differential Distribution of Erythroblasts.** A few studies have been concerned with the differential distribution of erythroblasts. It is difficult to interpret such material because of differences in terminology and lack of precise definition of the terms used. Tischendorf's material revealed erythroblastic hyperplasia with left shift of erythroblasts in all of his 11 patients.<sup>81</sup> Predominance of basophilic forms was mentioned by several authors.<sup>29, 47, 81</sup> According to Limarzi and co-workers the erythroblastic hyperplasia which occurred in their patients was due almost entirely to increase in the number of basophilic normoblasts, the pronormoblasts being significantly increased only rarely. Macronormoblasts were noted by Klima.<sup>47</sup> Isaacs described the marrow in uncomplicated cirrhosis as resembling that of pernicious anemia with megaloblasts present, but we have found no resemblance between the marrows of patients with pernicious anemia in relapse and cirrhosis of the liver. Our definition of the megaloblast has been given in detail elsewhere.<sup>11</sup> Specific denials of the presence of megaloblasts in the bone marrows of patients with cirrhosis of the liver has been made by Klima,<sup>47</sup> Limarzi et al.,<sup>81</sup> Ransier,<sup>72</sup> Benhamou,<sup>4</sup> Loeper and Vignatou,<sup>82</sup> Israels and Wilkinson,<sup>42</sup> and Fiessinger, Dupuy and Laur.<sup>79</sup>

**Granulocytes.** Granulocytic hypoplasia has been reported in patients with cirrhosis.<sup>1, 3, 31, 36</sup> as well as in rats with experimentally induced cirrhosis.<sup>82</sup> When complications such as infection or carcinomatosis are present or following laparotomy the myelogram may show leukopoietic hyperplasia.<sup>65, 84</sup>

\* Our term erythroblast denotes any nucleated red cell regardless of the state of maturation of either nucleus or cytoplasm.



Limarzi et al. observed an average of 26.3 per cent neutrophil myelocytes in the marrows of 20 patients. This represents an increase, as compared with our observations on normal persons.<sup>10</sup> Eosinophil leukocytes are sometimes increased in number even in the absence of eosinophilia in the peripheral blood.<sup>47-51</sup> The majority of reports do not mention alterations in the differential distributions of myeloid leukocytes.

**Lymphocytes.** The earlier studies of postmortem material yielded a few reports of increases of lymphocytes. However, it is unlikely that all the cells designated as small and large lymphocytes could be differentiated from other elements of lymphoid character because sectioned material was used. Rossier<sup>52</sup> described a decrease in the frequency of lymphoid nodules.

**Plasma Cells.** The frequency of plasma cells appears to be quite variable, some authors finding none<sup>53</sup> and others noting increases.<sup>47-51, 79-81</sup> Leitner<sup>80</sup> stated that in cirrhosis of the liver there is an increase of plasma cells, whereas the reverse is true of epidemic hepatitis.

**Reticulum Cells.** The evidence for or against reticulum cell hyperplasia is inconclusive. The usual aspiration technique is not satisfactory for determining the reticulum cell content of marrow, as these cells tend to be arranged in syncytial masses which are difficult to break up to form free cells in the aspirated fluid.<sup>6</sup> Even so, Rohr<sup>82-84</sup> and others<sup>47-50, 79-81</sup> found increased numbers of reticulum cells. Increased phagocytosis of pigment, usually hemosiderin, has been noted in many cases.<sup>81-83, 85-87</sup> Rohr remarked that there was no phagocytosis of fat in the reticulum cells in patients with fatty livers.<sup>83</sup>

**Qualitative Changes in Leukocytes.** Among the reported morphologic changes in the leukocytes are increased anisocytosis of granular elements<sup>79</sup> and vacuolization of monocytes and granulopoietic cells.<sup>81-83, 85</sup> It has been pointed out that the marrow of cirrhosis shows none of the peculiar disturbances of myeloid tissue seen in pernicious anemia,<sup>81</sup> but our material, as will be shown below, reveals that dysplasia of neutrophils, superficially resembling that seen in pernicious anemia, may occur.

**Megakaryocytes.** There are few recorded observations on megakaryocytopoiesis in cirrhosis of the liver, and the various reports are conflicting. This may be expected as the methods for estimating megakaryocyte content of aspirated marrow now in use are generally unsatisfactory.<sup>6-13</sup> Fiessinger, Dupuy, and Laur<sup>29</sup> found no megakaryocytes in the smears of sternal marrow from 10 patients, but Limarzi et al. reported an increased number in all of their 20 patients. Others<sup>1-5</sup> found normal or increased numbers and sometimes an increase of immature forms.

### *Results of the Present Study*

There is no evidence to show variations in the bone marrow picture which could be ascribed to differences in age or sex of adult patients, type, duration or severity of the liver lesion, or the type of anemia present, except that erythropoiesis may be slightly more active in patients with microcytic hypochromic anemia.<sup>4</sup>

**Fat and Cell Content of Marrow and ME Ratios.** Data concerning the relative volumes of fat and nucleated cells, as well as the differential distributions and ME ratios in our series of 25 patients are presented in table 4. The table also includes data from our control cases (19 normal individuals). The fat volumes showed a wide range, from 1 to 8 per cent, and a mean value of 1.8 per cent. The ME volumes ranged from 3 to 26.5 per cent, with a mean value of 13.9 per cent. Others<sup>51</sup> have considered the sternal marrows of patients with hepatic cirrhosis to be about twice as cellular as normal because of the finding of a mean ME volume of 13 per cent. Although this value is in close agreement with our findings in cirrhosis, our normal controls also yielded a mean ME volume of approximately 13 per cent. It has been shown, however, that the volumetric method provides only a crude estimate of relative fat and cell content of aspirated marrow and that it is not reliable for detecting small variations.<sup>8-9</sup> For these reasons, we made estimates of fat and cell content based on sectioned material, using the methods described by Berman and Axelrod.<sup>8-9</sup>

The results of this analysis are shown in table 5. There does not appear to be any large difference between the average relative fat or cell content of sternal marrow from cirrhotic and normal individuals, but the marrows of normal persons

TABLE 4.—*The fat volumes ME volumes ME ratios and differential distributions of nucleated cells in sternal marrow of 19 normal individuals and 25 patients with cirrhosis of the liver*

The data represent percentages. In each case the differential distribution is based on observation of a minimum of 2,000 cells. The symbol N in the first vertical column indicates the average counts on 19 normal persons.

Case	Fat volume	ME volume	ME ratio	Pronormoblasts	Basophilic normoblasts	Polychromatophilic normoblasts	Orthochromic normoblasts	Myeloblasts and leukoblasts	Promyelocytes (neut eos bas)	Neutrophil myelocytes	Neutrophil metamyelocytes	Band form neutrophils	Polymorphonuclear neutrophils	Eosinophils and basophils	Monocytes	Lymphocytes	Plasma cells	Reticulum cells	Macrophages	Case
N	2.1	13.1	2.1	3	10	86	1	3	9	6	9	31	17	2	5	14	1	2	Occ	N
1	3.0	4.0	2.1	4	10	85	1	2	15	6	13	25	13	3	4	14	1	2	1	1
2	1.5	4.0	1.1	2	8	87	3	3	11	6	15	29	5	1	4	22	4	1	1	2
3	1.0	6.0	2.1	4	6	76	13	5	7	7	14	27	15	5	4	10	5	1	Occ	3
4	1.0	12.0	1.1	1	9	90	Occ	8	10	5	13	27	12	2	3	13	6	2	2	4
5	1.5	25.0	2.1	2	5	91	2	1	7	8	13	29	18	2	4	10	4	2	1	5
6	1.0	22.0	1.1	2	5	89	5	3	7	8	15	19	13	2	6	17	Occ	5	Occ	6
7	1.5	9.5	2.1	3	9	86	1	4	10	7	12	19	19	3	4	15	2	4	1	7
8	2.0	16.5	3.1	3	10	87	Occ	4	11	7	14	28	20	2	2	8	3	1	Occ	8
9	1.0	3.0	4.1	2	5	92	1	5	11	5	12	25	17	2	7	12	Occ	1	1	9
10	1.0	4.5	3.1	1	7	91	Occ	2	7	3	12	25	23	2	6	15	3	1	Occ	10
11	1.0	11.0	1.1	2	10	87	Occ	4	16	4	13	27	12	3	5	11	2	1	2	11
12	—	—	1.1	2	6	88	3	3	22	3	13	25	10	1	3	13	3	4	1	12
13	1.0	3.5	2.1	2	8	90	Occ	3	5	3	9	24	20	2	3	28	1	1	Occ	13
14	2.0	9.0	3.1	1	8	90	1	2	7	2	8	29	26	1	6	12	Occ	6	Occ	14
15	1.0	19.0	1.3	2	10	88	0	3	12	5	10	25	11	3	6	17	2	4	1	15
16	4.0	22.0	2.1	2	10	87	Occ	1	15	7	16	26	8	1	3	12	7	3	Occ	16
17	1.0	24.0	3.1	3	17	79	Occ	5	27	4	10	24	9	2	3	10	3	2	Occ	17
18	1.0	15.0	2.1	3	12	85	Occ	2	15	4	13	25	20	2	4	10	2	3	Occ	18
19	1.0	6.0	2.1	2	9	85	3	6	11	4	12	24	12	4	6	17	2	1	Occ	19
20	—	—	3.1	3	9	86	1	2	7	5	12	30	20	2	3	13	2	3	1	20
21	2.0	12.5	1.1	2	8	89	1	4	8	7	16	23	17	2	4	10	5	4	Occ	21
22*	4.0	26.0	11.1	2	16	79	2	1	5	5	9	17	11	4	2	45	Occ	1	Occ	22*
23	0.5	13.0	1.1	3	9	87	1	10	15	5	16	23	6	3	6	12	Occ	3	1	23
24	8.0	19.0	2.1	3	6	89	2	2	8	2	9	23	26	2	6	16	2	4	Occ	24
25	2.0	22.0	1.1	2	6	91	1	3	10	7	15	27	8	3	5	16	2	3	Occ	25

\* Case 22 complicated by chronic lymphatic leukemia

are more likely to have fat contents over 30 per cent than is the case for patients with cirrhosis. The corollary is that the sternal marrows of patients with cirrhosis are likely to be more cellular than normal (table 6).

We found no relationship between the degree of cellularity of the marrow and the severity of the anemia. Hyperplasia of the marrow in cirrhosis occurred in spite

of absence of severe anemia. Hypocellularity is an unusual finding in the marrow of patients with this disease. In all except one case (case 25) the marrow was either normal or increased in cellularity, in spite of absence of signs of accelerated erythropoiesis in the peripheral blood. The conclusion made by Limarzi and co-authors regarding the common occurrence of hypercellularity of the sternal marrow is substantiated by our findings in sectioned material.

Analysis of patients with cirrhosis and of normal individuals as separate groups, as we have done above, may mask variations within the groups. Accordingly, we studied our data in an effort to detect possible relationships between the following groups of factors: fat content of bone marrow and fat content of the liver, ME volume and degree of anemia or severity of the liver lesion, ME ratio and degree

TABLE 5.—*Estimates of relative fat and cell content in sections of aspirated sternal marrow of patients with cirrhosis of the liver, and of normal individuals*

	% fat, range	% fat average	% cells range	% cells average
Normal	28-43	36	45-70	61
Cirrhosis	15-74	31	26-81	67

TABLE 6.—*Incidence of marrow with low fat and high cell content in patients with cirrhosis of the liver and in normal individuals as determined by study of sections of sternal marrow*

	Normal		Cirrhosis	
	Cases	Per cent	Cases	Per cent
Less than 30% fat	1	10	7	39
30% or more fat	9	90	11	61
Less than 70% cells	10	100	9	53
70% or more cells	0	0	8	47

of anemia, severity of the liver lesion or presence or absence of hemorrhagic phenomena.

The observation by Moosnick, Schleicher and Peterson<sup>40</sup> that choline therapy caused the fatty marrow and liver in a patient with refractory pernicious anemia to revert toward normal suggested a relationship between the fat content of these two organs. In our cirrhosis material there does not appear to be any quantitative relationship.

Low ME ratios were slightly, but not significantly, more frequent among patients with severe liver lesions. In general, patients who had experienced recent hemorrhages had lower ME ratios than the others. In one instance (case 15) a very low and reversed ratio of 0.4:1 occurred in a patient who had been bleeding from esophageal varices. Hence, recent blood loss increases the likelihood of a low ME ratio, which is expressive of relatively increased erythropoiesis, as would be expected, but hemorrhagic phenomena are not the sole contributing factors causing increased erythropoiesis in cirrhosis, as it was present also in patients without history or

evidence of bleeding. We agree with Fellingner and Klima that increased erythropoiesis in cirrhosis may be independent of bleeding.

*Differential Distribution of Erythroblasts* The differential distribution of erythroblasts in the marrow has been considered to be of diagnostic importance in hepatic cirrhosis. A review of the literature does not provide a clear picture of the type of differential pattern which may be expected. Morrison and Samwick<sup>62</sup> proposed an erythroblast-normoblast ratio which indicates the relationship between the number of early erythroblasts to the late erythroblasts (normoblasts). According to them a high erythroblast-normoblast ratio is indicative of disturbed liver function, and is suggestive of liver disease even before the clinical manifestations are apparent. We were not able to confirm this view as regards cirrhosis of the liver. The distributions of erythroblasts in patients with cirrhosis were within the normal range in our material (table 7).

*Qualitative Changes in Erythroblasts* Although no striking changes in the differential distributions of erythroblasts occurred, certain qualitative changes of diagnosis

TABLE 7—Differential distributions of erythroblasts in sternal marrows of 19 normal individuals and 25 patients with cirrhosis of the liver

	Norma		Cirrhosis	
	Range	Av	Range	Av
Pronormoblasts	1-5%	3%	1-4%	2%
Basophilic normoblasts	6-16%	10%	5-17%	9%
Polychromatophilic normoblasts	80-91%	86%	76-92%	87%
Orthochromatophilic normoblasts	0-4%	1%	1-13%	2%

tic significance were found. One patient with microcytic hypochromic anemia (case 2) exhibited a micronormoblastic marrow. The majority of the erythroblasts were small, and in the polychromatophilic stages the nuclei were generally pyknotic. The cytoplasm was poorly hemoglobinized and the cell contours were ragged. This is the type of erythroblasts we expect to find in instances of chronic iron deficiency anemia, as has also been stated by Scott.<sup>81</sup> In the remaining 24 patients, there were 11 (46 per cent) without evidence of qualitative change in the normoblasts, and 13 (54 per cent) with definite abnormalities.

The changes included increase of the diameters of normoblasts in all developmental stages, increase of nuclear diameters, and a disturbance of the nuclear-cytoplasmic ratios. The mean diameter of the large normoblasts was increased but the nuclear pattern, in most cases, was essentially that of normoblasts. The nuclear-cytoplasmic ratio was altered in favor of a relative increase of cytoplasm. The increase of cytoplasm was not as marked as seen in megaloblasts, and the large cells are of the type designated as macronormoblasts.<sup>45</sup> In a few patients, the nuclear structure in the early basophilic stages resembled that of reticulum cells. The reticular characteristics of the nuclei of these macronormoblasts persist throughout all stages of development. Without careful inspection it is easy to confuse such cells with megaloblasts of pernicious anemia, especially in the proerythroblast

stages. The differences between such abnormal large cells and megaloblasts of pernicious anemia have been emphasized by Jones<sup>46</sup> and Downey.<sup>25</sup> The frequency distributions and mean diameters of the polychromatophilic normoblasts in patients with cirrhosis and macronormoblastic marrows differ significantly from normal (fig. 1).

Theoretically, patients with cirrhosis of the liver might be expected to have megaloblastic marrow because of impaired storage of the antipernicious anemia principle, but no examples of such a change in erythropoiesis were seen in our series of 25 patients. Others have also stated that in the presence of cirrhosis of the liver the marrow is macronormoblastic in type.<sup>46</sup> Furthermore, the macrocytic anemias of cirrhosis of the liver do not respond to folic acid therapy in the manner of the macrocytic-megaloblastic anemias of the pernicious type.<sup>11</sup>

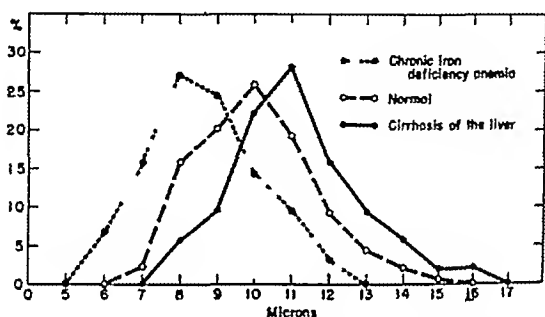


FIG. 1.—Typical frequency distribution curves of diameters of polychromatophilic erythroblasts in sternal marrow smears from patients with chronic iron deficiency anemia, cirrhosis of the liver with macrocytic anemia, and a normal individual. The mean diameters are 8.6, 11.1, and 10.0 microns, respectively.

We noted correlation between the presence of macrocytosis in the blood and macronormoblastic erythropoiesis in the marrow. Twelve of 13 patients (92 per cent) with macrocytic anemia had macronormoblastic marrow, whereas 5 of 11 patients (22 per cent) with normoblastic marrow had normocytosis in the peripheral blood, the others having only slight macrocytosis. The change to macrocytosis in the blood originates in dysplasia in the bone marrow, and is not related to factors acting on the red cells after they have entered the circulation.

**Granulocytes and Monocytes.** The differential distributions of the granulopoietic and monocytic cells showed remarkable constancy (table 4). Except in 2 patients (cases 12 and 17), there were no significant deviations from normal. One patient had an elevated percentage of neutrophil promyelocytes (21 per cent). This individual had neutrophilic leukocytosis and evidence of chronic cholecystitis at the time of the marrow aspiration. In the second patient the neutrophil promyelocytes comprised 25 per cent of the myeloid leukocytes. This patient had a marked neutrophilic leukocytosis, fever, and residual drainage following a rib resection eleven days previously, for empyema thoracis. The increases in promyelocytes were

compatible with the reactive change in myeloid leukopoiesis seen in some infections. Infection, as also observed by others,<sup>65-84</sup> may provoke reactive changes in leukopoiesis in patients with cirrhosis, but the differential distributions of myeloid leukocytes appear approximately normal in most cases, regardless of the presence of complications such as jaundice, fever, ascites or bleeding, all of which were present to varying extents among our patients. Others have pointed out the constancy of the marrow pattern in cirrhosis. Fiessinger, Dupuy and Laur<sup>29</sup> remarked that the myelogram was the same regardless of the appearance of icterus, oliguria, purpura, or the agonal state.

*Lymphocytes* In spite of the fairly regular appearance of lymphopenia in the peripheral blood, the marrows contained normal incidences of lymphocytes, except in one patient with 45 per cent lymphocytes (case 22) who had chronic lymphatic leukemia in addition to cirrhosis of the liver.

*Plasma Cells* The frequency of plasma cells is a point of special interest because of conflicting views in various reports. In our control material plasma cells comprise up to 2 per cent of the nucleated cells, exclusive of erythroblasts. A percentage of 4 per cent or more represents a significant increase. Six, or 30 per cent, of our patients had 4 per cent or more plasma cells in the sternal marrow smears. We are loth to accept Leitner's view that the plasma cell content of marrow may be of importance in the differential diagnosis of cirrhosis and epidemic hepatitis.

*Reticulum Cells* We do not regard the percentages of reticulum cells in smears of aspirated marrow as reliable indices of the reticulum cell content of sternal marrow. Such cells are best observed in imprints of marrow particles (Schleicher<sup>78</sup>), as they tend to remain fixed in the marrow tissue. There was no regular increase or decrease in the incidence of reticulum cells in our material, as observed either in the smears made from the first drop of aspirated marrow or from the concentrates of marrow, or from the imprints of marrow particles.

*Qualitative Changes in Leukocytes and Reticulum Cells* We were not able to confirm the previously reported prominence of vacuolization of granulocytes and monocytes in the marrows of patients with hepatic cirrhosis, such changes were of irregular appearance and never very marked. A few marrows revealed morphologic abnormalities of granulopoiesis. These included the appearance of giant band form neutrophils and giant polymorphonuclear neutrophils with hypersegmented nuclei. Such cells have a superficial resemblance to the large cells seen in pernicious anemia. However, there is no marked change in the type of granulation, nor is the chromatin pattern of the nuclei as fine as seen in the typical dysplastic cells associated with megaloblastic marrows. The significance of such changes is obscure. We have observed them occasionally in cases of iron deficiency anemia, in non-megaloblastic nutritional macrocytic anemias not responsive to liver extract therapy, leukemias, and carcinomatosis. The peculiar giant neutrophils were seen in three cases in our series (cases 12, 14, 24), and they were not numerous. We have mentioned them only because of their possible confusion with the characteristic macropolycytes, (Cooke<sup>17</sup>) or pernicious-anemia neutrophils (Jones<sup>42</sup>) of megaloblastic anemias.

In the few cases in which reticulum cells were relatively numerous (over 4 per

cent) the cells were of histiocytic rather than hematopoietic type. The nuclei were those of undifferentiated reticulum cells, and the cytoplasm was faintly acidophilic, abundant, and non-homogeneous, usually with some vacuolization and azurophil granulation, and often containing phagocytosed debris. We found no evidence of fat or lipid storage, but in 4 cases in which the liver contained relatively large amounts of iron-containing pigment, and especially in one case of pigmentary cirrhosis (case 16), the free reticulum cells contained large amounts of iron-containing pigment. The presence of macrophages with phagocytosed hemosiderin would therefore appear to imply that the liver also contains pigment in relatively large amounts.

*Megakaryocytes.* We have experienced difficulty in estimating the megakaryocyte content of aspirated marrow. The inadequacy of methods in use at present have been noted by various authors.<sup>6, 48, 53</sup> In our hands, the estimation of megakaryocytes based on their number in smears, as advocated by Limarzi et al.<sup>51</sup> has not yielded consistent results. Krumbhaar and Custer<sup>48</sup> have shown that reliable estimates can be based on enumeration of these cells in sectioned material. This is in accord with our experience. We have described our procedure for estimating

TABLE 8—*Megakaryocyte counts in normal individuals and in patients with cirrhosis of the liver*

	No. of cases	Range	Mean $\pm$ S. E.
Normals	10	14-60	35.7 $\pm$ 4.8
Cirrhosis	18	13-84	43.4 $\pm$ 5.8

\* Standard error of mean

megakaryocyte content in sectioned particles of aspirated marrow above, under the heading, Cases and Methods. The results in our series of patients are shown in table 8.

In the cirrhosis series, the megakaryocyte counts were slightly higher, on the average, than in the control series, but the difference was not statistically significant. The values obtained in cirrhosis were within normal limits or higher than normal. The point of chief interest is that, in spite of the common occurrence of peripheral thrombocytopenia, the megakaryocyte content of the marrow is normal or elevated in patients with cirrhosis of the liver. In this respect the findings are similar to those observed in instances of thrombocytopenia associated with hyperplasia. There were no important morphologic changes in the megakaryocytes nor changes in their differential distributions.

#### *Discussion of the Bone Marrow Findings*

The evidence from the literature and the information gained from the present study of the marrow in patients with cirrhosis of the liver may be summarized as follows:

1. Extension of red or functioning bone marrow into the shafts of the long bones is of regular occurrence in adults with cirrhosis of the liver.
2. The average relative fat content of the sternal marrow is not significantly

different from that of normal persons, but instances of high relative fat content are less likely to occur in patients with cirrhosis than is the case for normal individuals

3 The cellularity of the bone marrow in both the normal sites of hematopoiesis and in the sites representing extension of active marrow is normal or increased in most instances

4 Although hemorrhage tends to provoke marked relative increase of erythropoiesis, erythroblastic hyperplasia may be independent of bleeding in cirrhosis of the liver

5 The differential distribution of erythroblasts of patients with cirrhosis is not significantly changed, but there is a high degree of correlation between macrocytosis in the peripheral blood and the appearance of macronormoblastic erythropoiesis in the bone marrow. Megaloblastic erythropoiesis is rare, if present at all, in patients with hepatic cirrhosis uncomplicated by pernicious anemia

6 There are no significant changes in differential distributions of cells of the granulocytic, monocytic or lymphocytic series, but infection may result in a relative increase of immature granulocytes, especially promyelocytes. In occasional patients there is a disturbance of granulopoiesis indicated by the appearance of atypical giant neutrophils which are not identical with the characteristic macropolycytes or pernicious-anemia neutrophils associated with megaloblastic marrows

7 There are no constant quantitative changes in plasma cells or reticulum cells, although the finding of numerous reticulum cells containing hemosiderin implies hemosiderosis of the liver in patients with cirrhosis

8 Megakaryocytes are of normal or increased infrequency in the sternal marrows of patients with hepatic cirrhosis, but no qualitative changes of importance occur

It is clear that in patients with hepatic cirrhosis the marrow is of normal or increased cellularity, and that hypocellular marrows are unusual in spite of peripheral anemia which is often characterized by lack of signs of accelerated regeneration of red cells. Even in cases in which the sternal marrow is of normal cellularity, the fat in the shafts of the long bones is replaced by hematopoietically active tissue. With respect to cellularity, the most important change is extension of the marrow organ, since this appears to be more constant than hyperplasia at a given site, such as the sternum. In other words, the total active marrow in the body is increased in amount in cirrhosis of the liver

The fact that normal or relatively increased erythropoiesis is the rule, not only in marrow which is normally cellular, but in marrow which is usually fatty, is of interest because it occurs in spite of careful exclusion of blood loss. It has been suggested that the explanation may be that the patients with cirrhosis suffer a partial deficiency of the antipernicious anemia principle due to deficiency of storage or metabolism in the diseased liver, but the considerations which refute this hypothesis as an explanation for the peripheral macrocytosis apply to the problem of the marrow changes

Since it has been shown that patients with cirrhosis of the liver may excrete abnormally large quantities of urobilinogen,<sup>1 89</sup> we are inclined to believe that



the factor of excessive destruction of erythrocytes may play a role. The erythroblastic hyperplasia and the appearance of macro- and reticulonormoblasts in the marrow can be accounted for on the basis of hemolytic anemia, in which condition such types of erythroblasts are known to appear.<sup>7, 84</sup> The evaluation of the relative importance of hemolysis in the pathogenesis of the anemia is a problem worthy of further study.

The mechanism of hemolysis, if and when it occurs, must be regarded in the light of the concept of hypersplenism.<sup>13, 9, 91</sup> There are a number of peripheral blood and marrow changes which are suggestive of hypersplenism. The peripheral cytopenias (anemia and thrombocytopenia) occur in relation to normal or increased formation of erythroblasts and megakaryocytes in the marrow. These paradoxical phenomena are typical of hypersplenism which, in the case of cirrhosis, should be considered as manifestations of secondary hypersplenism. The well known involvement of the spleen in patients with hepatic cirrhosis is additional evidence in favor of this view.

#### *Diagnostic Significance of Hematologic Studies in Cirrhosis of the Liver*

Our hematologic studies, while controlled by observations on normal persons, have not been extended to other diseases which may be characterized by findings similar to those we have presented. Therefore we cannot consider any of the changes in blood and marrow we have described as pathognomonic of cirrhosis, even though they appear to be characteristic of the disease. This was emphasized by Tischendorf<sup>84</sup> who studied a group of patients with cirrhosis of the liver and other diseases of the liver, gall bladder and biliary tract. He felt that the sternal myelogram was not of value in the differential diagnosis of liver and gall bladder diseases, as some of the findings in these conditions are similar. On the other hand, it is not justifiable to consider complete blood and bone marrow studies as valueless and without diagnostic importance in cirrhosis of the liver.

For example, in patients known to have the disease, the appearance of microcytic hypochromic anemia or micronormoblastic marrow is indicative of chronic blood loss. The presence of hypocellular marrow in patients with macrocytic anemia suspected of having cirrhosis is unusual, and should point to other or additional factors in the clinical picture. Furthermore, although normocytic or macrocytic anemias are compatible with the diagnosis of cirrhosis, macrocytic hypochromic anemia, as determined by the mean corpuscular volume and mean corpuscular hemoglobin values, is not typical and should lead to further study of the patient. The marrow examination may be of crucial importance in distinguishing between pernicious or other megaloblastic anemias and the macrocytic anemia of cirrhosis, as the peripheral blood study does not provide evidence of the type of erythropoiesis in the marrow.<sup>11</sup>

Some patients with cirrhosis of the liver do not present unequivocal clinical signs of their disease. In such cases, we have found that the combined study of the peripheral blood and sternal bone marrow may lead the clinician toward serious consideration of cirrhosis of the liver. The combination of macrocytosis or macrocytic anemia without hypochromasia, plus lymphopenia and thrombocytopenia,

together with the presence of normal or increased marrow cellularity, and normal or increased erythrocytogenesis and megakaryocytopoiesis, constitutes a group of hematological findings which point strongly to cirrhosis of the liver. Furthermore, when anemia is absent and the other findings are present the probability of cirrhosis is even greater. An example of the latter type of case is given in the following brief case report.

*Case 25.* A 53 year old female was admitted for repair of a large abdominal incisional hernia. Systemic review was noncontributory. There was no history of alcoholism. Appetite and food intake were adequate. The preoperative blood protein and prothrombin levels were within normal limits. Because the liver and spleen were palpable the patient was referred for hematological survey. Serum bilirubin and urinary urobilinogen concentrations were normal. The bromsulphthalein test gave normal results. There was no anemia and the erythrocytes were normocytic and normochromic but lymphopenia (930 per cubic millimeter) and thrombocytopenia (57 000 per cubic millimeter) were present. The sternal marrow was hypercellular with a relative increase of erythrocytogenesis and a normal megakaryocyte count. The hematologic findings indicated a diagnosis of cirrhosis of the liver. A biopsy specimen obtained from the liver at the time of repair of the hernia revealed grade 2 cirrhosis.

We have found the combined blood and sternal marrow study useful in establishing the diagnosis of cirrhosis of the liver in patients in whom other diseases have obscured its manifestations, or in whom historical evidence was absent so that the clinical diagnosis was difficult to make.

#### SUMMARY

The peripheral blood and bone marrow findings in patients with cirrhosis of the liver have been analyzed on the basis of a review of the literature and the authors' study of 25 patients with diagnoses verified by biopsy of the liver.

The principal blood findings are macrocytic or normocytic anemia with normal or elevated mean corpuscular hemoglobin values, lymphopenia and thrombocytopenia in the majority of cases.

Anemia may be independent of bleeding, and the severity of anemia or macrocytosis does not appear to be related to the severity of the liver lesion.

The consistent change in the bone marrow is extension of the marrow organ so that active hematopoiesis is found in the shafts of the long bones.

Regardless of the presence or absence of bleeding or anemia, the marrow of the sternum is of normal or increased cellularity, with normal or increased erythrocytogenesis and megakaryocytopoiesis in most cases.

Hypocellularity of the marrow is an unusual finding, even in patients with advanced liver lesions.

Macronormoblastic erythropoiesis is seen in patients with macrocytic anemia, but megaloblastic erythropoiesis does not result from cirrhosis of the liver.

The presence of peripheral cytopenias (anemia and thrombocytopenia) in spite of normal or increased formation of erythroblasts and megakaryocytes in the marrow is suggestive of hypersplenism in patients with hepatic cirrhosis.

In patients with chronic hemorrhage the blood and bone marrow pictures are those of iron deficiency anemia, although other changes such as lymphopenia and thrombocytopenia tend to persist.

The combined peripheral blood and sternal marrow examination is often of value in establishing the diagnosis of cirrhosis of the liver

## APPENDIX

### Case Reports

*Case 1* White male age 61 with history of diabetes ascites two years Examination hepatosplenomegaly ascites edema of ankles no hemorrhagic phenomena RBC 2,790,000 Hb 7.8 grams MCV 91 MCH 28 WBC 5 500 platelets 144 000 Clinical impression hepatic cirrhosis, diabetes mellitus. Liver biopsy cirrhosis grade 3

*Case 2* White male age 55 with history of alcoholism ascites three weeks Examination hepatomegaly ascites spider angiomas loss of axillary and pubic hair no hemorrhagic phenomena RBC 3 710,000, Hb 9.3 grams, MCV 97 MCH 25 WBC 5 300 platelets 90,000 Clinical impression hepatic cirrhosis. Liver biopsy cirrhosis grade 4

*Case 3* White male age 45 with history of alcoholism ascites three weeks Examination hepatomegaly, ascites edema of ankles icterus spider angiomas, no hemorrhagic phenomena RBC 3 560,000 Hb 11.0 grams, MCV 96 MCH 31 WBC 11 000, platelets 150,000 Clinical impression hepatic cirrhosis. Liver biopsy cirrhosis grade 4

*Case 4* White female age 48 with history of alcoholism ascites and edema of ankles two weeks Examination hepatosplenomegaly ascites edema of lower extremities icterus spider angiomas no hemorrhagic phenomena RBC 3 760,000 Hb 10.9 grams MCV 90 MCH 29, WBC 12,500 platelets 200 000. Clinical impression hepatic cirrhosis Liver biopsy cirrhosis grade 4

*Case 5* Negro female age 31 hospitalized for meningitis from which uneventful recovery was made No history of alcoholism Examination hepatosplenomegaly RBC 2,970,000 Hb 10.1 grams MCV 121, MCH 34, WBC 6 650, platelets 220 000 Clinical impression probable hepatic cirrhosis but blood dyscrasia to be ruled out Liver biopsy cirrhosis grade 2.

*Case 6* White male age 52 with history of alcoholism icterus ascites edema of ankles two weeks Examination hepatomegaly ascites edema of ankles icterus no hemorrhagic phenomena RBC 3,070 000, Hb 10.7 grams, MCV 130 MCH 35 WBC 6 500 platelets 77,000 Clinical impression acute hepatitis superimposed on hepatic cirrhosis Liver biopsy cirrhosis grade 3

*Case 7* White male age 59 with history of alcoholism ascites and edema of lower extremities three weeks Examination hepatomegaly ascites edema of legs spider angiomas melena RBC 3 840,000 Hb 11.3 grams MCV 101 MCH 29 WBC 8 500 platelets 108 000 Clinical impression hepatic cirrhosis Liver biopsy cirrhosis, grade 3

*Case 8* White female age 31 with history of alcoholism hematemesis Examination hepatomegaly no hemorrhagic phenomena RBC 3 790 000 Hb 12.8 grams MCV 103 MCH 34 WBC 7 650 platelets 100 000 Clinical impression hepatic cirrhosis Liver biopsy cirrhosis grade 1

*Case 9* White male age 52 with history of alcoholism ascites one year Examination hepatosplenomegaly ascites no hemorrhagic phenomena RBC 3 700 000 Hb 11.5 grams MCV 97 MCH 30 WBC 3,350 platelets 111 000 Clinical impression hepatic cirrhosis Liver biopsy cirrhosis grade 1

*Case 10* White male age 38 with history of alcoholism icterus and weight loss five months Examination hepatosplenomegaly ascites icterus melena RBC 3 580 000 Hb 9.9 grams MCV 94 MCH 25 WBC 4 950 platelets 150 000 Clinical impression hepatic cirrhosis bleeding hemorrhoids Liver biopsy cirrhosis grade 1

*Case 11* White male age 59 with history of alcoholism icterus and weight loss four weeks Examination hepatosplenomegaly icterus melena RBC 4 200,000 Hb 12.6 grams MCV 98 MCH 28 WBC 10 450 platelets 230,000 Clinical impression acute hepatitis superimposed on hepatic cirrhosis terminal uremia Liver biopsy cirrhosis grade 2.

*Case 12* White female age 32 with history of alcoholism icterus followed by ascites four weeks Examination hepatomegaly ascites icterus spider angiomas fever no hemorrhagic phenomena RBC 2,590,000 Hb 8.7 grams MCV 135 MCH 25 WBC 11 350 platelets 126 000 Clinical impression hepatic cirrhosis chronic cholecystitis Liver biopsy cirrhosis grade 4

*Case 13* Negro male age 49 with history of alcoholism syphilis, icterus three weeks Examination

hepatomegaly slight icterus, no hemorrhagic phenomena RBC 4,950,000 Hb 15.4 grams, MCV 105 MCH 31, WBC 7,550, platelets 280,000 Clinical impression hepatic cirrhosis, hepatoma, or hepar lobatum Liver biopsy cirrhosis, grade 4, hepatoma

*Case 14* White male age 38 with history of alcoholism, weakness, nausea, vomiting icterus, ascites two months Examination hepatosplenomegaly ascites, edema of feet icterus spider angiomas, melena RBC 2,450,000 Hb 8.0 grams MCD 7.5 microns MCH 33 WBC 23,300 platelets 42,000 Clinical impression hepatic cirrhosis Liver biopsy cirrhosis grade 1 Autopsy cirrhosis, grade 1, acute suppurative pancreatitis

*Case 15* White male age 43 intermittent epistaxis, icterus weight loss five years Examination hepatosplenomegaly, ascites, icterus, melena RBC 3,870,000, Hb 8.4 grams MCV 72, MCH 22, WBC 4,850, platelets 94,000 Clinical impression biliary cirrhosis Liver biopsy cirrhosis grade 3

*Case 16* White male age 57 with diagnosis of cirrhosis of the liver and diabetes mellitus four years before present admission Examination hepatomegaly pigmentation of skin, no hemorrhagic phenomena RBC 3,810,000, Hb 12.8 grams MCV 100, MCH 34, WBC 10,500, platelets 160,000 Clinical impression hemochromatosis Skin biopsy hemochromatosis Liver biopsy pigmentary cirrhosis grade 4

*Case 17* White male age 62 with history of alcoholism diabetes mellitus seven years cough, weight loss one year Examination hepatomegaly spider angiomas, fever empyema thoracis, no hemorrhagic phenomena RBC 4,350,000, Hb 13.3 grams, MCV 95 MCH 31 WBC 24,500, platelets 143,000 Clinical impression hepatic cirrhosis empyema thoracis diabetes mellitus Liver biopsy cirrhosis grade 2.

*Case 18* White male age 46 mentally incompetent since infancy, anorexia, vomiting icterus one week Examination hepatomegaly, abdominal distension, icterus no hemorrhagic phenomena RBC 4,000,000 Hb 12.0 grams, MCV 100 MCH 30, WBC 10,800, platelets 321,600 Clinical impression infectious hepatitis Liver biopsy cirrhosis grade 1

*Case 19* White male age 59 with history of alcoholism, hematemesis, melena two weeks Examination hepatomegaly, ascites edema of ankles, loss of pubic hair melena RBC 3,610,000 Hb 9.9 grams, MCV 94 MCH 27, WBC 8,000, platelets 100,000 Clinical impression hepatic cirrhosis Liver biopsy cirrhosis, grade 4

*Case 20* White male age 68 with history of alcoholism, progressive weight loss ascites of unknown duration Examination ascites, no hepatosplenomegaly, no hemorrhagic phenomena RBC 4,730,000 Hb 12.0 grams MCV 97 MCH 25, WBC 12,050, platelets 288,000 Clinical impression hepatic cirrhosis Liver biopsy cirrhosis, grade 3

*Case 21* Negro male age 43 with history of alcoholism weight loss peri umbilical pain, nausea, three weeks Examination hepatosplenomegaly slight ascites icterus no hemorrhagic phenomena RBC 3,660,000, Hb 10.4 grams MCD 7.6 microns MCH 29 WBC 13,950, platelets 400,000 Clinical impression hepatic cirrhosis Liver biopsy cirrhosis, grade 2.

*Case 22* White female age 65 known to have had hypertension many years, progressive dyspnea and dependent edema of unknown duration Examination hepatosplenomegaly hypertensive retinopathy left ventricular enlargement, left pleural effusion no lymph node enlargement, no hemorrhagic phenomena RBC 5,000,000 Hb 14.7 grams MCV 94 MCH 29, WBC 47,200 platelets 201,000 Clinical impression hypertensive cardiovascular disease with decompensation chronic lymphatic leukemia Liver biopsy cirrhosis, grade 2. chronic lymphatic leukemia

*Case 23* White male age 71 with history of hematemesis ascites three years Examination hepatosplenomegaly esophageal varices, ascites RBC 2,750,000, Hb 7.0 grams, MCV 89 MCH 25 WBC 3,800 platelets 132,000 Clinical impression hepatic cirrhosis Liver biopsy cirrhosis grade 2.

*Case 24* White male age 58 with history of alcoholism progressive ascites edema weakness six years Examination hepatomegaly ascites spider angiomas no hemorrhagic phenomena coarse tremor left hand RBC 4,620,000 Hb 11.6 grams, MCV 91, MCH 25 WBC 5,900, platelets 193,000 Clinical impression hepatic cirrhosis Parkinsonism Liver biopsy cirrhosis grade 2.

*Case 25* White female age 53 with history of diverticulitis followed by peritoneal abscesses, colostomy, and colostomy repair two years before present admission Readmitted for repair of large incisional abdominal hernia Examination hepatosplenomegaly large abdominal hernia no hemorrhagic phenomena RBC 4,330,000 Hb 12.3 grams MCV 88 MCH 28 WBC 3,200 platelets 57,000 Clinical impression incisional hernia mild diabetes mellitus possible hepatic cirrhosis or blood dyscrasia. Liver biopsy cirrhosis grade 2.

## REFERENCES

- <sup>1</sup> ALESSANDRI, H., DUCCI, H., ETCHVEERRY, R. AND ALVAREZ, S. La anemia de la cirrhosis hepatica. Estudio del rol que en su determinismo juega el factor hemolisis. *Rev. méd. de Chile* 69: 634-638, 1941
- <sup>2</sup> ANDERSSON, A., AND STRANDELL, B. Lever cirrhos och anemi. *Nordisk Med* 3: 2961-2962, 1939
- <sup>3</sup> BENDER, J. cited by ALESSANDRI, H., ET AL. *Rev. méd. de Chile* 69: 634-638, 1941
- <sup>4</sup> BENHAMOU, E. Les anémies des cirrhotiques. *Presse méd* 45: 1027, 1937
- <sup>5</sup> —, AND NOUCHI, A. Les aspects du sang dans les cirrhotiques du foie. *Presse méd* 45: 1027-1030, 1937
- <sup>6</sup> BERMAN, L. Technics used in the study of aspirated sternal marrow. *Am. J. Clin. Path.* 17: 631-636, 1947
- <sup>7</sup> —. Unpublished data
- <sup>8</sup> —, AND AXELROD, A. R. Evaluation of volumetric data obtained by centrifugation of aspirated sternal marrow of adults. I. Estimation of relative fat content. *Am. J. Clin. Path.* 17: 551-556, 1947
- <sup>9</sup> —, AND —. Evaluation of volumetric data obtained by centrifugation of aspirated sternal marrow of adults. II. Estimation of cellularity of sternal marrow. *Am. J. Clin. Path.* 17: 557-560, 1947
- <sup>10</sup> —, AND —. The normal myelogram as revealed by sternal puncture. To be published
- <sup>11</sup> —, JACOBSON, S. D., AND VONDERHEIDE, E. C. Observations on response of macrocytic anemias to folic acid therapy. *Am. J. Clin. Path.* 16: 550-563, 1946
- <sup>12</sup> BIANCO, A., AND JOLIFF, N. The anemia of alcohol addicts. *Am. J. Med. Sc.* 196: 414-420, 1938
- <sup>13</sup> BLEICHMANN, cited by BLEICHRODER, F. Ueber Lebercirrhose und Blutkrankheiten. *Virch. Arch.* 177: 435-466, 1904
- <sup>14</sup> BLEICHRODER, F. Ueber Lebercirrhose und Blutkrankheiten. *Virch. Arch.* 177: 435-466, 1904
- <sup>15</sup> BORCH, J. N. Über Grösse, Volumen und Form der menschlichen Erythrozyten und deren Zusammenhang. IV. Über Makrozytose. *Wien. Arch. f. inn. Med.* 14: 219-256, 1927
- <sup>16</sup> CHENEY, G. The morphology of the erythrocytes in cirrhosis and other disorders of the liver. *California & West Med.* 39: 90-97, 1933
- <sup>17</sup> COOKE, W. E. The macropolycyte. *Brit. M. J.* 1: 12, 1927
- <sup>18</sup> DAMESHEK, W., AND MILLER, E. B. The megakaryocytes in idiopathic thrombocytopenic purpura, a form of hypersplenism. *Blood* 1: 27-30, 1946
- <sup>19</sup> DAVIS, W. D. Prognostic value of biopsy in cirrhosis of the liver. *Proc. Central Soc. Clin. Research* 29: 63-64, 1947
- <sup>20</sup> DOAN, C. A., AND WRIGHT, C. Primary congenital and secondary acquired splenic panhematopoiesis. *Blood* 1: 10-26, 1946
- <sup>21</sup> —, AND —. Further studies of the hypersplenic mechanism in human disease. *Proc. Central Soc. Clin. Research* 29: 8-9, 1947
- <sup>22</sup> DÖHNERT, H. R., AND TISCHENDORF, W. Die Blutzellbildung im Oberschenkelmark bei Lebercirrhose. *Folia haemat.* 58: 1-19, 1937
- <sup>23</sup> DOUGHERTY, T. F., AND WHITE, A. An evaluation of alterations produced in lymphoid tissue by pituitary-adrenal cortical stimulation. *J. Lab. & Clin. Med.* 32: 584-605, 1947
- <sup>24</sup> DOWNY, H. in E. T. Bell's *Textbook of Pathology*, ed. 2. Philadelphia: Lea & Febiger, 1938
- <sup>25</sup> —. Monocytic leucemia and leucemic reticulo-endotheliosis. in DOWNY, H. *Handbook of Hematology* vol. 2. New York: Paul B. Hoeber, Inc. 1938, P. 1321, case 7
- <sup>26</sup> EPPINGER, H. Die hepato-lienalen Erkrankungen. Berlin: J. Springer, 1920
- <sup>27</sup> FELLINGER, K., AND KLIMA, R. Untersuchungen über Anämien bei Lebercirrhosen. *Wien. klin. Wchnschr.* 46: 1191-1194, 1933
- <sup>28</sup> —, AND —. Lebercirrhose und Anämien. *Ztschr. f. klin. Med.* 126: 547-567, 1934
- <sup>29</sup> FISSINGER, N., DUPUY, R., AND LAUR, C. M. Les myélogrammes au cours des cirrhotiques. *Sang* 11: 88-95, 1936
- <sup>30</sup> GAMMA, C. L'anisocitosi dei globuli rossi del sangue nel suo significato patologico e diagnostico. *Minerva Med.* 6: 725-747, 1926
- <sup>31</sup> GARRETÓN, A. Estudio clínico de la cirrosis hepática de Laennec. *Rev. méd. de Chile* 69: 521, 1940

- <sup>22</sup> GLASS S J, EDMONSON, H A AND SOLL S N Sex hormone changes associated with liver diseases *Endocrinology* 27 749-752 1940
- <sup>23</sup> GOTTARDO, P, AND WINTERS W L Portal cirrhosis *Am J M Sc* 204 205-217, 1942
- <sup>24</sup> GRACIANSKY P, AND MNUZOV M La formule blanche dans les cirrhoses du foie *Presse méd* 54 665-667 1946
- <sup>25</sup> HEINLE, R W, CASTLE W B, AND ROSE F A Interpretation of the macrocytic anemia in experimental liver injury *Folia haemat* 64 174-189 1940
- <sup>26</sup> HERMOSILLA F, CERDA O, AND NEIRA M Les alteraciones hemáticas en las cirrosis del hígado *Rev méd de Chile* 68 579 1940
- <sup>27</sup> HIGGINS, G M AND STASNEY J Peripheral blood in experimental cirrhosis of the liver *Folia haemat* 54 129-144 1937
- <sup>28</sup> HILLER, G I HUFFMAN E R AND LEVEY S Plasma volume and total circulating proteins in cirrhosis of the liver From a paper read at the Regional Meeting of the American Federation for Clinical Research, March 5 1948, Detroit
- <sup>29</sup> HOFBAUER F W, EVANS, G T AND WATSON C J Cirrhosis of the liver with particular reference to correlation of comparative liver function studies with liver biopsy *Med Clin North America* 29 363-388, 1945
- <sup>30</sup> HOWAR B F Hemopathologic studies in liver disease *J Iowa M Soc* 28 148-150, 1938
- <sup>31</sup> ISAACS R The bone marrow in anemia The red blood cells *Am J M Sc* 193 181-191 1937
- <sup>32</sup> ISRAELS M C G, AND WILKINSON J F New observations on the aetiology and prognosis of achrestic anemia *Quart J Med* 9 163-176 1940
- <sup>33</sup> JONES, O P Origin of neutrophils in pernicious anemia (Cooke's macropolycytes) *Arch Int Med* 60 1002-1015 1937
- <sup>34</sup> — Cytology of pathologic marrow cells with special reference to bone marrow biopsies In DOWNER, H *Handbook of Hematology* vol 3 New York Paul B Hoeber 1938, Pp 2045-2101
- <sup>35</sup> — Morphologic, physiologic chemical and biologic distinction of megaloblasts *Arch Path* 35 752-775 1943
- <sup>36</sup> KINO, R B The blood picture in portal cirrhosis of the liver a report based on one hundred cases *New England J Med* 200 482-484 1929
- <sup>37</sup> KLIMA R Sternalpunktion und Knochenmarksbild bei Blutkrankheiten Berlin Urban & Schwarzenberg, 1938
- <sup>38</sup> KRUMBHAR E AND CUSTER R P A note on differential cell counts of bone marrow *Am J M Sc* 189 630-633, 1935
- <sup>39</sup> KUMPE, C W, GALL, E A, SCHIFF L, MULLER W E, SAIDI S A, AND STEINBERG, H H Needle biopsy of the liver I General considerations *Gastroenterology* 9 672-681 1947
- <sup>40</sup> LEITNER S J Die intravitale Knochenmarksuntersuchung Basel Bann Schwabe & Co 1945
- <sup>41</sup> LIMARZI, L R JONES R M PAUL J T AND PONCHER H G Sternal marrow in Banti's syndrome and other splenomegalic states *Am J Clin Path* 13 231-248 1943
- <sup>42</sup> LOEPER M, AND VIGNALOU J L anémie des cirrhotiques *Paris Méd* 109 297-303 1938
- <sup>43</sup> LOMBARDI, E Il comportamento del midollo osseo nelle cirrosi epatiche *Studium* 23 325-328 1933
- <sup>44</sup> MARKOFF N cited by TISCHENDORF W Über die Veränderung von Knochenmark und Blutbildung bei Leber und Gallenleiden *Deutsches Arch f klin Med* 182 261-287, 1938
- <sup>45</sup> MASINA M Das Blutbild bei Leberzirrhose mit besonderer Berücksichtigung der Mönchzytengranulation *Folia haemat* 46 335-345 1932
- <sup>46</sup> MCHARDY G BROWNE D C AND EDWARDS E Perimnenscopic and biopsy evaluation of hepatic disease *Gastroenterology* 9 682-685 1947
- <sup>47</sup> MEULENGRACHT cited by BOROS J N Über Grösse Volumen und Form der menschlichen Erythrozyten und deren Zusammenhang IV Über Makrozytose *Wien Arch f inn Med* 14 219-256, 1927
- <sup>48</sup> MICHELAZZI A M Osservazioni sul mielogramma e sulla formula eritrocitometrica periferica e midollare nella cirrosi epatica *Folia med* 23 975-985 1937
- <sup>49</sup> MONGES J, PINISO R AND FRUCTUS, A Les plaquettes sanguines chez les cirrhotiques *Marseille Med* 74 229-284 1937

- <sup>60</sup> MOOSNICK F B, SCHLEICHER, E M, AND PETERSON, W E Progressive Addisonian pernicious anemia, successfully treated with intravenous choline chloride J Clin Investigation 24: 278-282, 1945
- <sup>61</sup> MORLOCK, C G AND HALL, B E Association of cirrhosis, thrombopenia and hemorrhagic tendency Arch Int Med 72: 69-77, 1943
- <sup>62</sup> MORRISON, M AND SAMWICK A A Clinico-hematologic evaluation of bone marrow biopsies Am. J M Sc., 198 758-773, 1939.
- <sup>63</sup> NARGELI, O Blutkrankheiten und Blut diagnostik ed 5 Berlin, J Springer 1931
- <sup>64</sup> OSOOD, E. E. A Textbook of Laboratory Diagnosis, ed 3 Philadelphia The Blakiston Co 1940
- <sup>65</sup> PICENA J P La biopsia de la medula ossea Sus aplicaciones diagnosticas, pronosticas y terapeuticas Rev Med del Rosario 27: 1167-1354, 1937
- <sup>66</sup> PIZZOLATO, P, AND STASNEY J Quantitative cytologic study of multiple sternal marrow samples taken simultaneously J Lab & Clin Med 32: 741-748, 1947
- <sup>67</sup> REICH, C., AND KOLB ELIZABETH M. A quantitative study of the variations in multiple sternal marrow samples taken simultaneously Am J M Sci 204: 496-504 1942.
- <sup>68</sup> ROHR A. cited by TISCHENDORF, W Über die Veränderung von Knochenmark und Blutbildung bei Leber und Gallenleiden Deutsches Arch f klin Med 182: 261-287, 1938
- <sup>69</sup> — Das menschliche Knochenmark. Leipzig Georg Thieme 1940
- <sup>70</sup> — AND HAFTER, E. Untersuchungen über postmortale Veränderungen des menschlichen Knochenmarks Folia haemat 38: 38-50 1937
- <sup>71</sup> ROSENBERG, D H, AND WALTERS, A Macrocytic anemia in liver disease particularly cirrhosis Observations on the incidence course and reticulocytosis with a correlated study of the gastric acidity Am J M. Sc. 192: 86-98 1936
- <sup>72</sup> ROSSIER, J cited by FIERSINGER N ET AL. Les myélogrammes au cours des cirrhoses Sang 11: 88-95 1936
- <sup>73</sup> RÖSSLER cited by STASNEY, J, AND HIGGINS G M. The blood-forming organs in experimental cirrhosis of the liver Folia haemat 35: 93-107 1936
- <sup>74</sup> RUSSO G Rilievi ematologica nelle cirrosi epatiche. Riv di Clin Med 36: 1-28 1935
- <sup>75</sup> SARAORA, T, AND SEICARESCO MME Contributions à l'étude morphologique du sang dans les cirrhoses Bull et Mém Soc Méd d Hôp d Bucarest 8: 271-276 1926
- <sup>76</sup> — AND — Contributions à l'étude morphologique du sang dans les cirrhoses ascitogènes Bull et Mém Soc Méd d Hôp d Bucarest 8: 277-283, 1926
- <sup>77</sup> SCHIFF L., RICH, M L. AND SIMONS, S D The haematopoietic principle in the diseased human liver Am J M Sc., 196 313 1938
- <sup>78</sup> SCHLEICHER, E M. The morphology of the erythrocytogenic reticulum of the bone marrow in Addison Biermer's disease in relapse and early remission as seen by the imprint method. J Lab & Clin Med 30: 928-943, 1945
- <sup>79</sup> SCHULTEN, H Die Sternalpunktion als diagnostische Method Leipzig Georg Thieme, 1937
- <sup>80</sup> SCHUK, H, AND LÖWY H Über das Verhalten des Knochenmarks in Krankheiten und seine Beziehungen zur Blutbildung Ztschr f klin Med 40: 412-463 1900.
- <sup>81</sup> SCOTT, R B Sternal puncture in diagnosis of diseases of the bloodforming organs Quart. J Med 8: 127-172, 1939
- <sup>82</sup> STASNEY, J, AND HIGGINS G M The blood forming organs in experimental cirrhosis of the liver Folia haemat 35: 93-107, 1936
- <sup>83</sup> THADDEA, S cited by LEITNER S J Die intravitale Knochenmarksuntersuchung Basel Benno Schwabe & Co 1945
- <sup>84</sup> TISCHENDORF, W Über die Veränderung von Knochenmark und Blutbildung bei Leber und Gallenleiden Deutsches Arch f klin Med 182: 261-287, 1938
- <sup>85</sup> VAUGHAN, S L., AND BROCKMYRE FRANCES Normal bone marrow as obtained by sternal puncture Blood, Special Issue No 1 July 1947 pp 54-59
- <sup>86</sup> VOLWILER, W Studies on chronic hepatitis by needle biopsy Bull New England Med Center 9: 161-165, 1947
- <sup>87</sup> —, AND JONES, C. M. Diagnostic and therapeutic value of liver biopsies with particular reference to trocar biopsy New England J Med 237: 651, 1947

- <sup>11</sup> WAHI, P N , AND CHAKRAVARTI, M G Study of liver diseases Correlation of clinical and liver function studies with liver biopsy *Indian M Gaz* 81 454-458, 1946
- <sup>12</sup> WATSON, C J Studies of urobilinogen III The per diem excretion of urobilinogen in the common forms of jaundice and diseases of the liver *Arch Int Med* 59 206-231 1937
- <sup>13</sup> — Hemolytic jaundice and macrocytic hemolytic anemia certain observations in a series of 35 cases *Ann Int Med* 12 1782-1796 1939
- <sup>14</sup> WHITBY L. E H AND BRITTON C J C Disorders of the Blood, ed 5 Philadelphia, The Blakiston Co , 1947
- <sup>15</sup> WILSON, T E Sternal biopsy *M J Australia* 1 405-410 1940
- <sup>16</sup> WINTROBE M M Clinical Hematology ed 2 Philadelphia Lea & Febiger 1946
- <sup>17</sup> — AND SHUMACKER H S The occurrence of macrocytic anemia in association with disorder of the liver together with a consideration of the relation of this anemia to pernicious anemia *Bull Johns Hopkins Hosp* 52 387-407 1933
- <sup>18</sup> WOLONIK cited by BLERICHROEDER, F Ueber Lebercirrhose und Blutkrankheiten *Virch Arch* 177 435-466 1904
- <sup>19</sup> WRIGHT D O Macrocytic anemia and hepatic cirrhosis *Am J M Sc* 189 115-119, 1935
- <sup>20</sup> ZYRKIN, S M Zur Pathogenese der Bluterkrankungen *Virch Arch* 174 103-193 1903



# AN EVALUATION OF STERNAL ASPIRATION AS AN AID IN DIAGNOSIS OF THE MALIGNANT LYMPHOMATA

By TALBERT COOPER, M D , AND CHARLES H WATKINS, M D

THE DIAGNOSIS of malignant lymphoma can be definitely established only by histologic examination of the tissue involved. In the majority of instances palpably enlarged superficial lymph nodes are present and properly performed biopsy provides the correct diagnosis in a direct and relatively simple manner. However, in a significant number of cases of Hodgkin's disease, lymphosarcoma and follicular lymphoma the primary site of involvement is thoracic, abdominal or some otherwise inaccessible location for simple biopsy.

Symmers<sup>1</sup> has stated that in Hodgkin's disease primary enlargement of abdominal or of abdominal and thoracic nodes combined is ten times more common than primary enlargement of the cervical nodes. Ewing<sup>2</sup> supported this view, emphasizing that the superficial nodes which first attract attention may be only the outlying manifestations of an internal lesion. Jackson and Parker<sup>3</sup> found the superficial nodes primarily involved in all of 26 cases of Hodgkin's paraganuloma, but in only 8 of 59 cases of Hodgkin's granuloma, and in none of 27 cases of Hodgkin's sarcoma. Sugarbaker and Craver<sup>4</sup> regarded the primary site of involvement as extranodal in approximately one third of 196 cases of lymphosarcoma. In 14.2 per cent of cases in which lymph nodes were involved primarily, the site of origin was abdominal or mediastinal. These observations contrast clearly with the common impression that superficial lymph nodes, particularly those of the cervical area, constitute the usual site of primary involvement in these conditions.

Biopsy of superficial nodes, then, may be of no value in establishing a diagnosis in some cases and of value only after the disease process is well advanced in others.

This study was undertaken in an attempt to evaluate the clinical usefulness of aspiration of sternal bone marrow as a method for obtaining material of diagnostic significance in cases of malignant lymphoma.

## REVIEW OF THE LITERATURE

### *Bone or bone marrow involvement in cases of malignant lymphoma*

*Hodgkin's disease.* The dominant histologic change in Hodgkin's disease involves the reticular cells of the reticulo-endothelial system. The lymphatic elements do not take an active part in the hyperplasia in most cases and often are diminished in number.<sup>5</sup> It might reasonably be expected that an organ rich in reticulo-endothelial tissue as is the bone marrow would be commonly involved in the disease. Steiner,<sup>6</sup> in his comprehensive review of the subject, suggested that bony lesions might develop in one of three ways (1) by direct invasion from contiguous

From the Division of Medicine, Mayo Clinic, Rochester, Minn.

Abridgment of thesis submitted by Dr. Cooper to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Master of Science in Medicine.

lymphogranulomatous masses, (2) by hematogenous spread or (3) by primary origin in the marrow

Krumbhaar<sup>7</sup> reported a case of Hodgkin's disease of the bone marrow and spleen without apparent involvement of lymph nodes. Herscher<sup>8</sup> and Livingston<sup>9</sup> observed cases in which, at autopsy, the process appeared to be confined to the bones and liver.

The reported incidence of bone or bone marrow involvement in Hodgkin's disease is highly variable, particularly in studies based on clinical evidence alone. In summarizing the reported series in which the diagnosis of bone or bone marrow involvement was based on clinical grounds, Steiner<sup>6</sup> determined an average incidence of 8.3 per cent (166 of 2,006 cases). Lesions of bone most frequently involving the spinal column and pelvis, were noted during life in 23 per cent of Jackson and Parker's<sup>3</sup> 133 cases of Hodgkin's granuloma. Similar lesions were detected in 14.8 per cent of 257 cases of Hodgkin's disease reported by Vieta and co-workers.<sup>10</sup> The latter authors called attention to cases of extensive marrow involvement discovered at postmortem examination in which the same bones had appeared normal on previous roentgenologic examination. The degree of cortical involvement appears to determine to a considerable extent the roentgenographic appearance of bone, and extensive medullary lesions may not be detected by this method.

According to Ewing,<sup>11</sup> bone marrow lesions, both typical and atypical, form a prominent feature in many cases of Hodgkin's disease, at times they may dominate the clinical course and it is rare that a thorough search at autopsy fails to disclose some deposits in the bone marrow. Steiner<sup>6</sup> noted an average incidence of 28.3 per cent of bony lesions in 547 reported autopsies but observed that the incidence reported from any series apparently depended on the thoroughness of the skeletal examination. He studied microscopic sections taken at random from 3 to 9 easily accessible bones in 14 cases of Hodgkin's disease and found marrow lesions in 78.6 per cent of cases. The vertebrae, pelvis, ribs, femur, sternum, skull and humerus were most commonly involved. Sixty-three and seven-tenths per cent of sternal sections contained lymphogranulomatous lesions. Steiner<sup>6</sup> further observed that there was no basis for the impression that skeletal lesions occur only as a late manifestation of Hodgkin's disease.

*Lymphosarcoma.* By original definition,<sup>12</sup> lymphosarcoma lacks the systemic character of Hodgkin's disease and leukemia. Arising as an apparently local change in lymphadenoid tissue, it seems to extend by local invasion, by continuous growth through lymph channels and by the formation of true metastatic lesions in distant organs.

Lymph vessels have not been demonstrated in bone marrow but small accumulations of lymphatic tissue along the small arteries have been described by most investigators.<sup>13</sup> Lymphosarcoma might, then, arise in the bone marrow but this structure would seem no more likely to become secondarily involved than would any other organ.

Sugarbaker and Craver<sup>4</sup> noted clinical evidence of bony involvement in 9.7 per cent of 196 cases of lymphosarcoma. In 1 per cent, the process appeared to arise in bone marrow. Vieta and co-workers<sup>10</sup> found roentgenologic evidence of bony

involvement in 7 per cent of 213 cases, while on postmortem examination lesions were noted in 29 per cent of 54 cases. The authors regarded the latter figure as probably too low since the examinations at necropsy were limited to easily accessible bones and the skeletal examination was sometimes omitted entirely. Lesions of bone in this series were most often a late manifestation of the disease. Such lesions appeared in only 22 per cent of cases during the first half of the course of the illness while in 63 per cent the bony involvement appeared to develop during the terminal one third of the illness. By contrast, in cases of Hodgkin's disease observed by the same authors, 37 per cent of the bony lesions were clinically evident before the first half of the course of the disease had elapsed. It was further observed that while the bony lesions in lymphosarcoma were, as in Hodgkin's disease, most common in the bones rich in red marrow there was a tendency for a more generalized distribution of lymphosarcomatous lesions to occur.

The less detailed observations of other authors<sup>11-14-18</sup> would indicate that bone marrow involvement in lymphosarcoma occurs infrequently and then as a manifestation of a late, generalized stage of the disease.

*Follicular lymphoma* Sugarbaker and Craver<sup>4</sup> have regarded follicular lymphoma as a setting for lymphosarcoma since later biopsies in several of their cases have shown the development of typical reticulum cell lymphosarcoma. Whatever the exact relationship between the two processes may be, it appears to be an intimate one.

Gall and co-workers<sup>19</sup> noted bony lesions (clinically evident) in 6 of a series of 63 cases of the follicular type of malignant lymphoma.

#### *Reported clinical experience with sternal aspiration in cases of malignant lymphoma*

*Hodgkin's disease* Young and Osgood<sup>20</sup> found that study of specimens of aspirated sternal marrow was of no diagnostic value in 2 cases of Hodgkin's disease. Vogel and co-workers<sup>21</sup> observed a slight left shift and, in a few cases, an increase in eosinophils and reticulum cells. It was noted that 3 of the 5 patients studied had received intensive irradiation therapy during the year preceding the examination of the sternal marrow. Émile-Weil and Perlès<sup>22</sup> obtained negative or inconclusive results in most cases. In 10 of 25 instances, medullary hyperplasia was noted. A slight increase in polymorphonuclear neutrophils, eosinophils, plasma cells and monocytes was commonly observed. Although no Reed-Sternberg cells were noted the authors suggested that the differentiation of such cells from megakaryocytes would be difficult. Paraf and co-workers<sup>23</sup> reported 1 case in which a sternal tumor was present. Sternal aspirations at three sites yielded material suggesting only erythromyeloid aplasia with lymphocytes and plasma cells predominant. After study of the findings in 14 cases Falconer and Leonard<sup>24</sup> observed the sternal marrow in this group in some instances showed a leukemoid or myeloid reaction difficult to distinguish from the early myeloid reaction of myelogenous leukemia. In only 1 case was the specimen of sternal marrow the basis for the diagnosis of Hodgkin's disease, a trephine specimen which revealed fibrosis was obtained in this case.

Scott<sup>25</sup> examined specimens in 8 cases and concluded that the findings, while

dependent on the stage of the disease, were variable and nonspecific. In none were Reed-Sternberg cells found. In 3 there were varying degrees of myeloid left shift. In 2 some increase in number of megakaryocytes was noted. One patient presented aplastic changes which were attributed to previous irradiation therapy. Baras-cutti<sup>26</sup> reported 6 cases in which marked eosinophilia of the marrow occurred. Mendell and co-workers<sup>27</sup> noted no characteristic changes in specimens of marrow from 3 patients. Propp and Schwind<sup>28</sup> stated that myelophthisic anemia such as occurs in Hodgkin's disease and reticulosis are among the diseases giving marrow pictures which are not diagnostic. Piney and Hamilton-Paterson<sup>29</sup> stated that we probably never obtain assistance in diagnosis by examining the bone marrow in Hodgkin's disease and described hyperplastic and hypoplastic changes in varying stages of the disease. Sundberg<sup>30</sup> and Wintrobe<sup>31</sup> agreed that the usually encountered marrow picture is nonspecific, consisting of some shift to the left in the myeloid line together with a slight monocytosis or eosinophilia. Limarzi<sup>32</sup> reported myeloid hyperplasia, an increase in plasma cells, histiocytes and megakaryocytes in some cases of Hodgkin's disease.

While the majority of investigators have found aspiration of sternal marrow of little value as a diagnostic procedure in Hodgkin's disease, there are a few exceptions. Váradi<sup>33</sup> reported a single case in which sternal aspiration yielded a specimen containing many lymphocytes and large basophilic cells with large nuclei and large, blue nucleoli which he classified as Reed-Sternberg cells. Rohr and Hegglin<sup>34</sup> identified Reed-Sternberg cells in the specimen of marrow in a case of Hodgkin's disease. Klima<sup>35</sup> described a lymphogranulomazellen, which he felt to be a derivative of the lymphoblast, as the characteristic cell of Hodgkin's disease. Scott<sup>36</sup> regarded the cell described by Klima as a partly differentiated reticulum cell such as is frequently seen in imprint and puncture preparations from lymph nodes of many conditions other than Hodgkin's disease. From 1 patient Sundberg<sup>30</sup> described section preparations showing the granulomatous lesions typical of Hodgkin's disease, but only after an extremely diligent search was a single Reed-Sternberg cell found in smear preparations from the same patient.

*Lymphosarcoma.* Dameshek and co-workers<sup>36</sup> in illustrating the comparative value and limitations of trephine and simple aspiration methods of sternal marrow biopsy reported 2 cases of lymphosarcoma. On attempted aspiration no cells were obtained in 1 and very few cells in the other case. Study of sections obtained by the trephine method established the diagnosis in each case, disclosing lymphosarcomatosis with connective tissue replacement of the marrow in the first, and a small area of lymphoblastic proliferation (lymphosarcoma) in the second. Vogel and co-workers<sup>37</sup> reported the marrow findings essentially normal in 4 cases of lymphosarcoma and in 2 of follicular lymphoma. Falconer and Leonard<sup>38</sup> found that study of aspirated marrow material was of no aid to diagnosis in 4 cases of lymphosarcoma. Wintrobe<sup>31</sup> noted an increase of lymphocytes in some cases of lymphocytic lymphoma and in 1 of follicular lymphoma, but more commonly he found no abnormality in the marrow in such cases. Gormsen,<sup>39</sup> in 2 of 18 cases of lymphosarcoma, observed moderate infiltration of the sternal marrow with more or less immature lymphatic elements.

## MATERIAL AND METHODS

*Material* The material for this study was obtained by simple needle aspiration of sternal bone marrow in 15 unselected cases of Hodgkin's disease, 10 of lymphosarcoma and 2 of follicular lymphoma. The diagnosis in each case was based on results of lymph node biopsy, autopsy or both.

Because of the uniformly poor results reported following examination of simple smears after needle aspiration, no cases were included in this series in which examination was carried out prior to the introduction, for routine use in this laboratory, of the methods of preparation advocated by Schleicher.<sup>28, 29</sup>

*Technic* With the Illinois sternal aspiration needle\* a total of approximately 2 cc of sternal marrow substance was aspirated. The specimen was transferred immediately to a paraffin-lined container and mixed gently with a minute pinch of heparin powder as an anticoagulant.

Portions of the material obtained were used for preparation of the usual smears, Wright's stain (Grübler) being used and for volumetric determinations. The latter procedure has been found to provide a fairly accurate quantitative index of the functional state of the marrow.

The grossly visible particles of marrow substance, or units, in the aspirated specimens were carefully collected. These units ranged from 0.5 to 1.0 mm in diameter in the normal individual to as much as 0.3 to 4.0 mm in the hyperplastic marrow of pernicious anemia. Several of these units were speared on the tip of a wooden applicator and the material smeared out gently on the surface of a glass slide. The resulting imprint preparations provided a picture of the general structural relationships of the marrow.

Finally, after fixation, the remaining units were stained with hematoxylin and eosin and section preparations obtained. Sections so prepared provide architecturally and histologically accurate samples of the marrow. With this method, it has been felt that the needle aspiration method more closely approaches a true biopsy procedure, and the advantages of the trephine method have been, to a considerable extent, overcome. It was hoped that aspirated material so prepared would yield information of diagnostic significance in circumstances in which the simpler aspiration and smear techniques had reportedly failed.

*Plan of study* The preparations described above were carefully examined. Differential counts of 1,000 nucleated cells were carried out in each case. The clinical features in the cases under consideration were analyzed and some correlation with the appearance of the marrow specimen was attempted.

## RESULTS OF STUDY

*Hodgkin's disease*

*Criteria for diagnosis* Hodgkin's disease of the bone marrow exhibits the same histologic picture seen in other involved tissues and organs. Hyperplasia of reticular cells is often the dominant change.<sup>3</sup> However, the process is characterized by

\* Manufactured by the V. Mueller Co., Chicago, Ill.

pleomorphism and the diagnosis rests finally on the demonstration of the presence of Reed-Sternberg cells, whether the pathologic change be paraganulomatous, granulomatous or sarcomatous in type.<sup>2</sup>

Piney and Hamilton-Paterson<sup>29</sup> have stated that there is no certain way of distinguishing Reed-Sternberg cells from megakaryocytes. While it is true that the differentiation may be difficult, it is felt that it can be satisfactorily accomplished in most instances if undistorted, properly stained cells are considered.

The mature cells are similar in size. The nuclei of Reed-Sternberg cells are round, oval, lobulated, multilobed or multinucleated. The nuclear chromatin is relatively

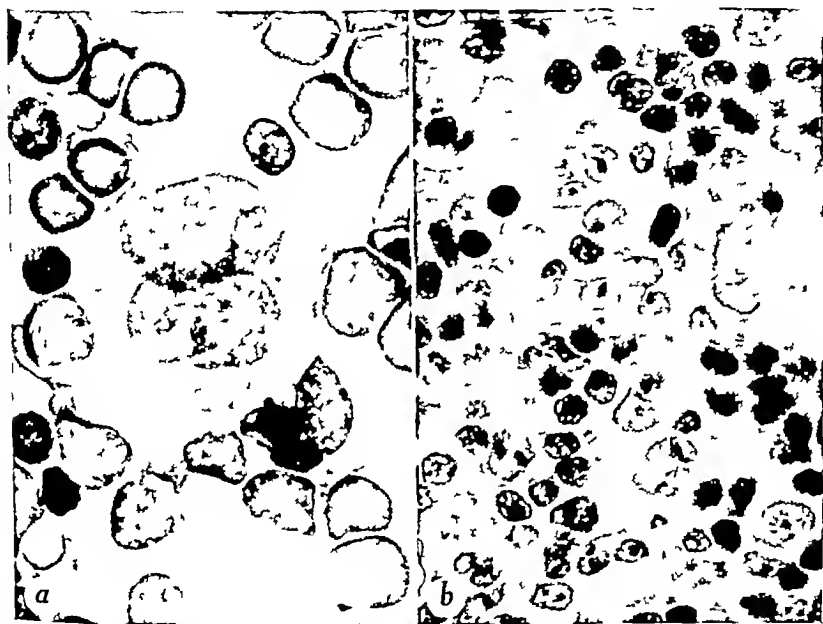


FIG. 1—REED-STERNBERG CELLS. Imprints of lymph nodes from a patient with Hodgkin's disease. *a* Wright's stain  $\times 970$ . *b* Hematoxylin and eosin stain  $\times 970$ .

scanty in amount and irregularly distributed (fig. 1). Megakaryocytic nuclei, although often multilobed, are always single with generous, more uniformly distributed chromatin and a fine chromatin-parachromatin pattern. The outstanding characteristic of the Reed-Sternberg cell is the very prominent nucleolus (fig. 1*b*) which is usually lacking in the megakaryocyte or megakaryoblast. The cytoplasm of the normal megakaryocyte contains characteristic azurophilic granulation when stained with the polychrome dyes. In addition, pseudopodia with apparent platelet formation are often observed. The generous cytoplasm of the Reed-Sternberg cell has a faintly basophilic, granular appearance with Wright's stain and the cell membrane is often indistinct.

**Results.** Detailed search of all material obtained by sternal aspiration in the

proved cases of Hodgkin's disease in this series revealed no Reed-Sternberg cells. Satisfactory section preparations were obtained in 9 instances but in none were lesions suggestive of Hodgkin's disease demonstrable.

In 6 instances, the specimen of marrow appeared normally active, and in 5 there was distinct hyperplasia. Other variations from normal in this group were minor in degree and of nonspecific character, consisting of myeloid preponderance with slight shift to the left in 5, mild eosinophilia in 3, toxic changes in cells of the myeloid series in 4 and diminished erythrogenic activity in 3. One or more of these changes was present in each case.

In the remaining 4 cases, attempted aspiration resulted in a relatively dry tap, although a few drops of marrow were obtained from which smears were made. Such a result with this procedure in the hands of an experienced individual should, per se, raise suspicion of disease of the marrow. It has occurred most often in cases of myelofibrosis, acute leukemia and metastatic carcinoma involving the bone marrow. In Hodgkin's disease the attempted aspiration is probably defeated by the fibrous character or hypercellular consistency of the involved tissue. In this connection, Loseke and Craver<sup>10</sup> experienced difficulty in obtaining satisfactory or sufficiently large specimens in 11 of 25 cases of Hodgkin's disease in which needle aspiration of lymph nodes and other involved tissues was attempted. Smear preparations in 1 of the 4 dry tap cases revealed few normal marrow elements with small lymphocytes composing 91 per cent of the nucleated cells, in the remaining 3 cases there was an increase in number of morphologically normal lymphocytes with moderate reduction in number of erythrogenic and myeloid cells. It should be emphasized that despite the negative findings on sternal aspiration, demonstrable bone or bone marrow involvement was present in 4 cases of this series. The dorsolumbar vertebrae were the site of the clinically evident lesions in 3 while in the remaining case the clavicles and several ribs were involved.

No correlation could be established between the marrow findings and the duration of the disease, the apparent degree of dissemination of the process, the amount of previous irradiation therapy or the peripheral blood picture. A mild to moderately severe anemia, hypochromic in type, was noted in 11 of the 15 cases in this group. The anemia was accompanied by evidence of active regeneration of erythrocytes, including the presence of macrocytes and polychromatophilia, in 9 instances. Monocytosis was noted in 6, myeloid immaturity in 4 and eosinophilia in 3.

*Comment.* No consistent abnormalities or diagnostically specific changes were encountered in the study of sternal marrow material in this series of cases of Hodgkin's disease.

The occurrence of dry taps in 4 instances was regarded as suggestive of marrow involvement but careful examination of the smears made from the meager specimens obtained disclosed nothing of diagnostic significance.

The lesions of the bone marrow in Hodgkin's disease may be focal and of microscopic proportions or extensive and grossly demonstrable.<sup>6</sup> When small, focal lesions exist, chance alone might account for disappointing results on attempted needle aspiration. Aspiration of marrow material from several sternal sites, from vertebral bodies, and perhaps, from the iliac crest might enhance the diagnostic po-

tentialities of the procedure. In addition, the sternum should be routinely palpated for areas of tenderness and such localities should be selected as the site for aspiration.

The fibrosis which so commonly develops in the lesions of Hodgkin's disease could conceivably render simple aspiration of a satisfactory specimen impossible. Utilization of the trephine method, in dry tap cases particularly, might overcome this difficulty.

The high incidence of positive findings on sections taken at random post mortem by Steiner<sup>6</sup> would indicate that more frequent positive results should follow the adoption of the proper technic. This should be particularly true in patients presenting clinical<sup>41</sup> or hematologic evidence of bone marrow involvement.

### *Lymphosarcoma*

*Criteria for diagnosis.* No one cell has been shown to be diagnostic of lymphosarcoma. Ghon and Roman<sup>42</sup> emphasized the usual presence of a mixture of cells and commented that lymphosarcoma appears to be a neoplasm in which all elements of the normal lymph node may be represented. These cells ranged from typical small lymphocytes through larger, atypical cells with indented, hyperchromatic nuclei and relatively little cytoplasm, to lymphoblastic cells with reticular nuclear structure, sometimes containing nucleoli, and a basophilic, often vacuolated, cytoplasm. Lymphocytic, lymphoblastic and reticulum cell varieties of lymphosarcoma have been commonly described.<sup>14</sup> Gall and Mallory<sup>43</sup> subdivided lymphosarcoma into stem cell, clasmatocytic, lymphoblastic and lymphocytic types, according to the predominant cell type. Hellwig<sup>44</sup> has advanced a similar classification.

Sternberg,<sup>45</sup> however, described a cell which he regarded as characteristic of lymphosarcoma, occurring in cases of so-called leukosarcoma. Sternberg considered the cell a form of lymphocyte but, at the same time, an atypical tumor cell. The majority of hematologists have not accepted leukosarcoma as an entity and prefer to consider it a locally aggressive type of leukemia, most often large cell and acute in type.<sup>16</sup>

Isaacs<sup>46</sup> noted the development of leukocytosis in 15 of 43 cases of lymphosarcoma. He described a characteristic cell appearing in the peripheral blood in those cases of lymphosarcoma cell leukemia which he felt was usually mistaken for an immature lymphocyte or lymphoblast. Certain distinguishing features of the nucleoli were stressed. When stained with Wright's stain after the material has been smeared on cover slips treated with cresyl blue, the nucleolus stands out as a sky blue, round area surrounded by a deep blue-black rim of cytoplasm which is piled up around it. Such nucleoli were usually single. In contrast, nucleoli of immature lymphocytes or lymphoblasts appeared as a light blue hole in the chromatin structure, without the heavily staining rim. In addition, the chromatin around the edge of the nucleus of the lymphosarcoma cell was thickened into a fairly definite nuclear wall. In 6 of the 15 cases in this group necropsy disclosed transformation, in varying degrees, of all lymphoid tissue in the body into the lymphosarcoma type. The autopsy findings cited in these cases would seem more consistent with the diagnosis of leukemia than lymphosarcoma.



Wiseman<sup>47</sup> said it is possible, by use of vital staining methods, to differentiate normal, leukemic and lymphosarcomatous lymphocytes

Gall and Mallory<sup>48</sup> considered the development of a leukemic blood picture an incidental manifestation of the underlying neoplastic process in lymphosarcoma. Blood pictures resembling leukemia occurred at some time in the course of the disease in 18 per cent of the lymphocytomas and in 28 per cent of the lymphoblastomas reviewed by Hellwig.<sup>49</sup> Evans and Leucutia<sup>48</sup> advanced the concept that lymphosarcoma becomes leukemia when the bone marrow is involved.

Webster<sup>49</sup> regarded lymphosarcoma, lymphatic leukemia and leukosarcoma as manifestations of the same disease. There appears to be little doubt that the processes are closely related, and absolute differentiation is commonly difficult and sometimes impossible.

In addition to the doubtful existence, according to the majority of investigators, of a cell characteristic of the disease, the positive microscopic diagnosis of lymphosarcoma is further complicated in that the general histologic picture may be closely simulated in other conditions, notably Hodgkin's sarcoma and lymphatic leukemia. Potter<sup>50</sup> suggested that the diagnosis of small cell lymphosarcoma should be eliminated from consideration in lymph node enlargements and labeled leukemia.

*Results of study.* Material permitting satisfactory section, imprint and smear preparations was obtained in all cases in this group, however, the technical difficulty encountered was sufficient to warrant the designation dry tap in 2 instances.

In 3 cases the specimen of marrow presented no remarkable deviation from the normal. Lymphocytosis, ranging from 30.6 to 70.9 per cent with an average of 46.2 per cent, was present in the remaining 7 cases. The lymphocytosis was accompanied by moderate to marked diminution in number of erythrogenic cells. In contrast to the frequent finding of myeloid hyperplasia with left shift in cases of Hodgkin's disease, such changes were not observed in this group.

The fixed sections presented the most spectacular findings. In 3 instances the marrow was infiltrated or invaded by obviously abnormal tissue composed of mononuclear cells (fig. 2). The picture presented was one of focal involvement, with apparently uninvolved marrow tissue interspersed. This contrasts with the usual appearance of the marrow in lymphatic leukemia (fig. 3) in which, while nodules of lymphocytes may be present, the involvement is usually more diffuse in character. This difference may not be striking on superficial examination (figs. 3a and 4a) but on closer study cells of the myeloid and megakaryocytic series can be identified even in a densely infiltrated marrow in chronic lymphatic leukemia (fig. 3b). On the other hand, no normal marrow elements can be identified among the lymphocytic cells composing the infiltrate in cases of lymphosarcoma (fig. 4). Whether this distinction will be sufficiently consistent to be regarded as definitely diagnostic can be determined only by study of more cases.

Fixed sections in the remaining cases appeared normal in 3 instances and presented varying degrees of hypoplasia, without aggregations of mononuclear cells, in 4.

In 3 cases there was no significant deviation from the normal either in the morphologic character of the lymphocytes or in other features observed on the smear preparations. Atypical and abnormal lymphocytic types were present in all 7 cases in which there was some degree of lymphocytosis in the marrow specimen. No single lymphocytic type predominated in these cases but rather a variety of forms was encountered on the smear preparations. The prevailing types could be loosely separated into the following categories:



FIG. 2.—LYMPHOSARCOMATOUS INFILTRATION OF BONE MARROW. Section preparation (Hematoxylin and eosin stain  $\times 90$ ).

**Type 1.** This was a large (10 to 18 micra) round to oval cell containing an irregularly shaped, frequently indented nucleus with relatively scanty, basophilic cytoplasm (fig. 5a). The dense chromatin material was uniformly distributed with little parachromatin evident. Distinct nucleoli were fairly numerous.

**Type 2.** Similar in size (14 to 18 micra) to the cells described as type 1, this cell (fig. 5b) demonstrated less bizarre nuclear configuration and more generous, basophilic cytoplasm. The nuclear structure was reticular with frequent grooving and occasional indistinct nucleoli. A clear perinuclear zone was occasionally observed.

**Type 3.** These cells (fig. 5b) were 8 to 12 micra in diameter and presented dense, hyperchromatic, frequently grooved, occasionally Rieder-type nuclei with a very

thin rim of deeply basophilic cytoplasm. This was the abnormal cell type most commonly encountered.

**Type 4** These cells (fig 5c), measuring 14 to 20 micra in diameter, appeared much like normal large lymphocytes but contained smoothed out, irregular shaped, often eccentrically placed nuclei with rare indistinct nucleoli. The cytoplasm was sky blue in color and presented occasional azure granules.

These atypical or abnormal cells were seen in company with varying proportions of lymphocytes having a morphologically normal appearance. While a single type of abnormal cell was usually predominant in each case a mixture of types was most

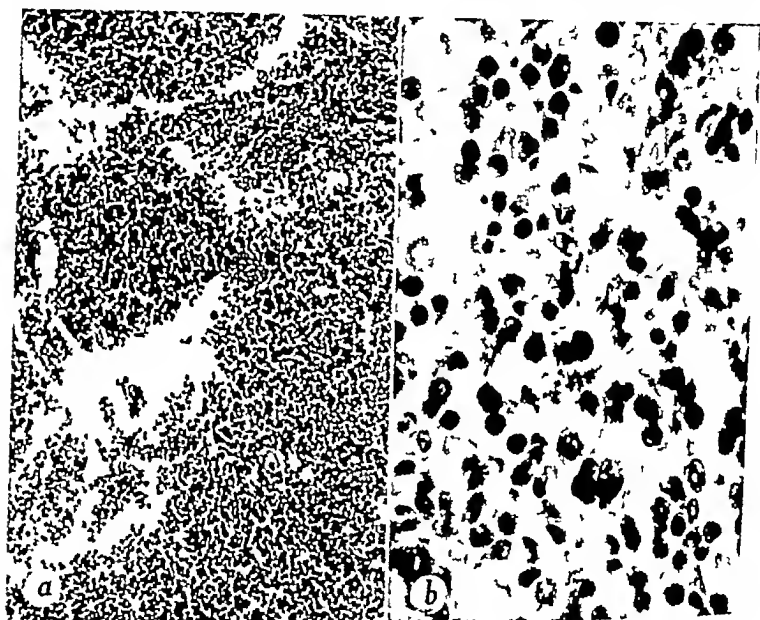


FIG 3 —SECTION PREPARATIONS OF MARROW FROM A PATIENT WITH LYMPHATIC LEUKEMIA STAINED WITH HEMATOXYLIN AND EOSIN *a*  $\times 105$  *b*  $\times 700$

commonly encountered (fig 5b). No correlation could be made in this series between the type of cell predominating in the sternal smear and the type according to the morphologic classification advanced after biopsy and autopsy.

The cell types observed might be confused with, or may indeed be identical with, atypical or bizarre forms sometimes encountered in subacute or acute lymphatic leukemia but are clearly distinguishable from the ordinary lymphocyte or lymphoblast.

In 5 of the 7 cases in which atypical lymphocytes were demonstrated in the marrow specimen, similar cells were observed in smears of the peripheral blood. In 4 of these, including the 3 cases in which there were positive fixed sections, peripheral lymphocytosis ranging from 36 to 64 per cent was noted at some time

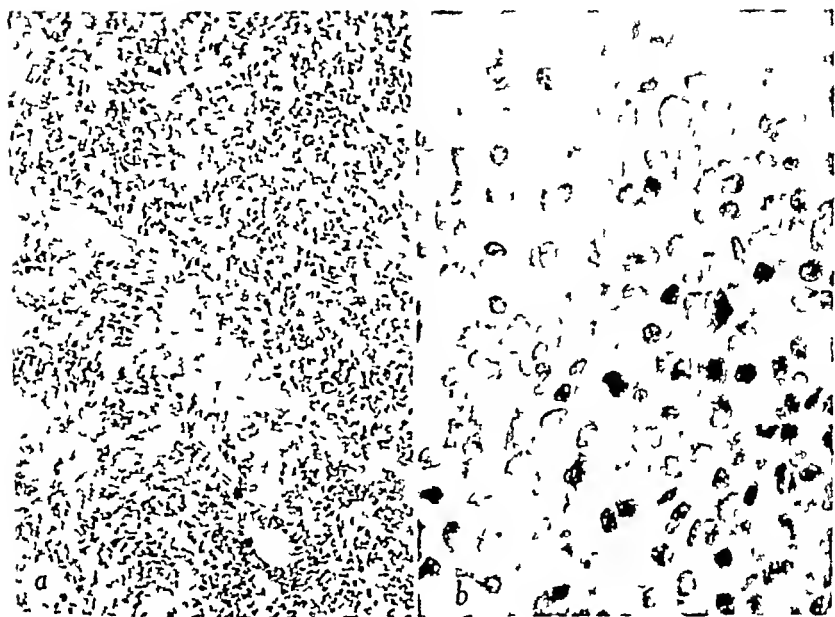


FIG 4—LYMPHOSARCOMATOUS INFILTRATION OF MARROW Section preparations stained with hematoxylin and eosin *a*  $\times 140$  *b*  $\times 760$



FIG 5—SMEAR PREPARATIONS OF MARROW FROM A PATIENT WITH LYMPHOSARCOMA STAINED WITH WRIGHT'S STAIN *a* All type 1 predominates ( $\times 675$ ) The section preparation is shown in figure 2 *b* Cell types 2 and 3 are illustrated Section preparation is shown in figure 4 ( $\times 720$ ) *c* Cell type 4 is illustrated ( $\times 750$ )

# STERNAL ASPIRATION IN MALIGNANT LYMPHOMATA

during the period of observation. The total leukocyte count per cubic millimeter of blood ranged from 3,700 to 17,500 for the entire group.

No other abnormalities were consistently noted on study of the peripheral blood in these cases although minor degrees of myeloid immaturity and erythrocytic regeneration were occasionally observed. Despite the diminution in number of erythrogenic cells commonly noted (7 cases) on examination of sternal specimens, mild normocytic anemia occurred in only 2 cases.

No clinical evidence of bony involvement was noted in this group. In all cases superficial lymph nodes were palpably enlarged, the lymphadenopathy involving the anterior cervical, axillary and inguinal groups with approximately the same frequency. The spleen was palpably enlarged in 60 per cent. In no case was remarkable hepatomegaly demonstrated. The duration of symptoms prior to sternal aspiration ranged from two months to five years with the average duration of illness being shorter (eleven months) in the patients presenting most marked changes in the bone marrow. A history of previous irradiation therapy was elicited in 4 cases but could not be correlated with the findings noted on examination of the marrow in these cases.

*Comment.* The high incidence of abnormal findings in this group of cases was surprising. It is felt that the demonstration of lymphocytic tumor infiltrates in the bone marrow should have the same diagnostic significance as the same finding in a lymph node or other tissue would have. The greatest difficulty will probably be experienced in histologically differentiating this picture from that of lymphatic leukemia.

While the specific diagnostic significance of the abnormal cell types encountered in 7 of the 10 cases in this group must be further evaluated, their presence in the bone marrow or peripheral blood would appear to justify the suspicion that lymphosarcoma exists.

## Follicular lymphoma

This condition, which appears to be closely related to lymphosarcoma, is characterized histopathologically by the development in lymphoid tissue of multiple, follicle-like nodules of variable size.<sup>21</sup> The predominant cell type in such a process has been described as an ordinary lymphocyte or lymphoblast.<sup>14, 43, 51</sup>

In 2 cases reported by Baggenstoss and Heck,<sup>51</sup> later biopsies revealed the picture of lymphosarcoma.

Satisfactory specimens were obtained in the 2 cases composing this group. In 1 instance there was a slight increase in number (20 per cent of nucleated cells) of morphologically normal lymphocytes. In the other, a hyperplastic specimen with preponderance of the myeloid line was obtained. Neither presented features of diagnostic significance.

In each, the spleen and superficial lymph nodes were moderately enlarged. The marrow lymphocytosis noted in the first case was not reflected in the peripheral blood which, but for a mild normocytic anemia, appeared normal. In the second case, leukopenia (1,400 leukocytes per cubic millimeter of whole blood) with a relative lymphocytosis and monocytosis was present. The patient had recently

completed a course of irradiation therapy before examination at the clinic. Symptoms had developed twelve to eighteen months prior to sternal aspiration. No clinical evidence of bony involvement was demonstrated in either case.

*Comment* While sternal aspiration in these cases provided no information of diagnostic significance it is felt that, in view of the close relationship between follicular lymphoma and lymphosarcoma, study of a larger series of cases may well reveal more significant changes.

### SUMMARY AND CONCLUSIONS

Neither diagnostically significant features nor consistent abnormalities of other character were demonstrated in the specimens of sternal marrow obtained in 15 cases of Hodgkin's disease. With improvements in technic, particularly in patients presenting clinical evidence of bone or bone marrow involvement, the procedure might become more valuable.

As an aid in diagnosis in cases of obscure malignant lymphoma, sternal aspiration is likely to prove of greatest value in cases of lymphosarcoma. In 7 of 10 proved cases, abnormal lymphocytic cell types were encountered and in 3 instances bone marrow infiltrations were demonstrated in fixed section preparations. The latter were felt to be diagnostic of lymphosarcoma.

In 2 cases of follicular lymphoma the specimens of sternal marrow presented no striking abnormalities. However, because of the apparently close relationship which this disease bears to lymphosarcoma it is felt that study of a larger number of cases may prove the procedure of some diagnostic value.

### REFERENCES

- <sup>1</sup> SYMMERS, DOUGLAS. The clinical significance of the pathological changes in Hodgkin's disease. *Am J M Sc* 167 157, 313 1924.
- <sup>2</sup> EWING J. Discussion. *J Cancer Research* 8 517 1924.
- <sup>3</sup> JACKSON, H., JR. AND PARKER F., JR. Hodgkin's disease: pathology. *New England J Med* 231 35, 1944.
- <sup>4</sup> SUGARBAKER, E. D. AND CRAVER L. F. Lymphosarcoma: a study of 196 cases with biopsy. *J. A. M. A.* 115 17 112, 1940.
- <sup>5</sup> JAFFÉ, R. H. The Reticulo-endothelial System. In DOWNEY H. A. L. *Handbook of Hematology*, vol. 2. New York: Paul B. Hoeber Inc. 1938. Pp. 977-1271.
- <sup>6</sup> STEINER P. L. Hodgkin's disease: the incidence, distribution, nature and possible significance of the lymphogranulomatous lesions in the bone marrow, a review with original data. *Arch Path* 36 627 1943.
- <sup>7</sup> KRUMBHAR E. B. Hodgkin's disease of bone marrow and spleen without apparent involvement of lymph nodes. *Am J M Sc* 182 764 1931.
- <sup>8</sup> HERSCHER HARRY. Hodgkin's disease of bone marrow and liver without apparent involvement of lymph nodes. *Am J Roentgenol* 35 73 1936.
- <sup>9</sup> LIVINGSTON, S. K. Hodgkin's disease of skeleton without glandular involvement, a case report proved by autopsy. *J Bone & Joint Surg* 17 189 1935.
- <sup>10</sup> VIETA J. O. FRIEDELL H. L. AND CRAVER L. F. Survey of Hodgkin's disease and lymphosarcoma in bone. *Radiology* 39 1 1942.
- <sup>11</sup> EWING JAMES. *Neoplastic Diseases: A Treatise on Tumors*, ed. 4. Philadelphia: W. B. Saunders Co. 1940. 1160 pp.
- <sup>12</sup> KUNDRAT H. Quoted by WATSON C. J.<sup>13</sup>
- <sup>13</sup> MAXIMOW A. A. AND BLOOM WILLIAM. *A Textbook of Histology*, ed. 4. Philadelphia: W. B. Saunders Co. 1942. 695 pp.

- <sup>14</sup> BOYD WILLIAM A Text-book of Pathology ed 4 Philadelphia, Lea & Febiger, 1943 1008 pp
- <sup>15</sup> WATSON, C J Lymphosarcoma and leucosarcoma, interrelations of leucemia and diseases of the pseudoleucemia group (Hodgkin's disease aleucemic lymphadenosis, lymphosarcoma) In DOWNEY HAL Handbook of Hematology vol 4 New York Paul B Hoeber, Inc., 1938 pp. 3051-3106
- <sup>16</sup> DUVAL, C. W AND SCHATTENBERG, H J Textbook of Pathology, A Correlation of Clinical Observations and Pathological Findings New York D Appleton-Century Company, Inc., 1939 681 pp.
- <sup>17</sup> WOOD, F C Delafield and Prudden's Text-book of Pathology, ed 16 Baltimore, William Wood & Co 1936 1406 pp
- <sup>18</sup> MACCALLUM W G A Textbook of Pathology, ed 7 Philadelphia, W B Saunders Company, 1940. 1301 pp
- <sup>19</sup> GALL, E. A., MORRISON H R., AND SCOTT, A T The follicular type of malignant lymphoma, a survey of 63 cases Ann Int Med 14 2073 1941
- <sup>20</sup> YOUNG R H., AND OSGOOD, E E. Sternal marrow aspirated during life cytology in health and in disease Arch Int Med 55 186 1935
- <sup>21</sup> VOGEL, PETER, ERF, L. A AND ROSENTHAL, NATHAN Hematological observations on bone marrow obtained by sternal puncture Am J Clin Path 7 436, 498 1937
- <sup>22</sup> ÉMILE WEIL, P AND PERLÈS SUZANNE La ponction sternale, procédé de diagnostic cytologique. Paris Masson & Cie, Éditeurs 1938 183 pp
- <sup>23</sup> PARAY, J FISCHGOLD, H., ABASA, A., AND LEWIS, S Maladie de Hodgkin à localisation osseuse et cutanée (données de la ponction sternale) Sang 13 797, 1939
- <sup>24</sup> FALCONER, E H AND LEONARD M E The value of sternal marrow aspiration as a method of bone marrow biopsy Ann Int Med 15 446 1941
- <sup>25</sup> SCOTT R B Sternal puncture in the diagnosis of diseases of the blood forming organs Quart J Med. 8 127, 1939
- <sup>26</sup> BARASCUTTI, A Quoted by SCOTT R B <sup>25</sup>
- <sup>27</sup> MENDRELL, T H., MERANZE D R AND MERANZE, THEODORE The clinical value of sternal bone marrow puncture Ann Int. Med 16 1180 1942
- <sup>28</sup> PROPP, SIMON, AND SCHWIND J L. Sternal puncture as a practical diagnostic procedure. Ann Int. Med 21 580 1944
- <sup>29</sup> PINEY, A., AND HAMILTON PATERSON, J L. Sternal Puncture, A Method of Clinical and Cytological Investigation, ed 3 New York Grune & Stratton Inc., 1946 80 pp
- <sup>30</sup> SUNDBERG, R DOROTHY Sternal aspiration Bull Staff Meet Hosp Univ Minn 17 389 1946
- <sup>31</sup> WINTROBE, M M Clinical Hematology ed 2 Philadelphia, Lea & Febiger 1946 862 pp
- <sup>32</sup> LIMARZI L. R The diagnostic value of sternal marrow aspirations. Illinois M. J 75 38, 1939
- <sup>33</sup> VÁRADY STEPHÁN L infiltration lympho-granulomateuse du sternum Sur un cas de maladie de Hodgkin diagnostiquée par ponction sternale Sang 12 106 1938
- <sup>34</sup> ROHR, A. AND HEGOLIN H Tumorzellen in sternal Punktar Metastasennachweis maligner Geschwülste im Knochenmark Deutsches Arch f klin Med 179 61, 1936
- <sup>35</sup> KLIMA, R Quoted by SCOTT R B <sup>25</sup>
- <sup>36</sup> DAMESHEK, WILLIAM, HENSTELL, H H AND VALENTINE ELEANOR H The comparative value and the limitations of the trephine and puncture methods for biopsy of the sternal bone marrow Ann Int Med 11 801, 1937
- <sup>37</sup> GORMSEN, H Demonstration of tumor cells in sternal punctures of patients with bone metastases. Nord Med (Hospitalstud) 14 1310 1942.
- <sup>38</sup> SCHLEICHER E M Method for making imprints and direct smears from gross marrow units Am J Clin Path (Tech sect) 9 8 1945
- <sup>39</sup> ——— Volumetric pattern of aspirated normal human sternal marrow of males 18 to 40 years Am J Clin Path 14 370, 1944
- <sup>40</sup> LOSEKE, L., AND CRAVER, L. F Diagnosis of Hodgkin's disease by aspiration biopsy Blood 1 76, 1946
- <sup>41</sup> WOODARD H Q AND CRAVER, L. F Serum phosphatase in the lymphomatoid diseases J Clin Investigation 19 1, 1940
- <sup>42</sup> GHON, A., AND ROMAN B Quoted by WATSON, C. J <sup>15</sup>

- <sup>40</sup>GALL, E. A., AND MALLORY T. B. Malignant lymphoma: a clinico-pathologic survey of 618 cases. *Am. J. Path.* 18: 381, 1942.
- <sup>41</sup>HELLWIG, C. A. Malignant lymphoma, analysis of 202 cases. *Am. J. Clin. Path.* 16: 564, 1946.
- <sup>42</sup>STERNBERG, C. Quoted by WATSON C. J.<sup>18</sup>
- <sup>43</sup>ISAACS, RAPHAEL. Lymphosarcoma cell leukemia. *Ann. Int. Med.* 11: 657, 1937.
- <sup>44</sup>WISEMAN, B. K. Lymphopoiesis, lymphatic hyperplasia and lymphemia: fundamental observations concerning the pathologic physiology and interrelationships of lymphatic leukemia, leukosarcoma and lymphosarcoma. *Ann. Int. Med.* 8: 1303, 1936.
- <sup>45</sup>EVANS, W. A., AND LEUCUTIA, T. The neoplastic nature of lymphatic leukemia and its relation to lymphosarcoma. *Am. J. Roentgenol.* 15: 497, 1926.
- <sup>46</sup>WEBSTER, L. T. Lymphosarcoma, lymphatic leukemia, leukosarcoma, Hodgkin's disease. *Bull. Johns Hopkins Hosp.* 31: 458, 1920.
- <sup>47</sup>POTTER, EDITH L. Hodgkin's disease with special reference to its differentiation from other diseases of lymph nodes. *Arch. Path.* 19: 139, 1935.
- <sup>48</sup>BAGGENSTOSS, A. H., AND HECK, F. J. Follicular lymphoblastoma (giant lymph follicle hyperplasia of lymph nodes and spleen). *Am. J. M. Sc.* 200: 17, 1940.



## BONE MARROW REGENERATION IN EXPERIMENTAL BENZENE INTOXICATION

By BERNHARD STEINBERG, M D

**B**ENZENE intoxication is known to affect the hemopoietic system of man and of lower animals. Depending upon the duration of exposure, concentration of the chemical, frequency of administration and the age of the animal, changes of the bone marrow vary from complete aplasia to selective hypoplasia. Since little is known of the regulating mechanism of production and distribution of leukocytes, the action of hemopoietic intoxicants may be utilized for the study of this problem. This work was undertaken from that point of view.

The mechanism by which benzene induces bone marrow changes has not been determined. Two possibilities appear plausible in the present state of our knowledge. The chemical may inhibit cell division or interfere with hypothetic active principles concerned with marrow activity. Cell division may be inhibited either by damage to the nucleus or by the alteration of the lipid-protein medium of the marrow in which the cells are contained. The latter process presupposes that the *cell metabolism requires the integrity of the medium*.

### EXPERIMENTAL PROCEDURE

This study is a morphologic investigation of the effect of benzene on cell division. The following experimental procedures were employed for the evaluation: (1) Marrow of one or more of the long bones was extirpated in living rabbits according to a procedure described previously.<sup>1</sup> (2) Various degrees of benzene intoxication were induced in rabbits. (3) The animals were killed at intervals after varying periods of benzene administration. (4) Studies were made of the comparative changes between the extirpated and the controlateral unextirpated marrows. (5) Comparative changes were studied between extirpated marrow of normal animals and those with benzene intoxication. The steps in regeneration of extirpated marrow in normal animals were presented in a previous publication.<sup>2</sup>

Extirpation of marrow was done by incising the soft tissues at each end of the long bone. In the removal of tibial marrow the two incisions are preferable. At the narrow end of the bone, the tendons were retracted. At the broad end a cross incision was made to the periosteum. In the case of the femur, humerus, radius and ulna, a single incision from the proximal to the distal end is sufficient. The muscles were separated along fascial lines and were retracted. Muscle injury is the common cause of death of the animals. A single opening was made at the narrow end and four openings at the broad end with a Rask drill. The piece of bone outlined by the four openings was lifted out. A tight-fitting flexible silver cannula was inserted into the single opening. A syringe filled with sterile liquid petrolatum was attached to the cannula. The pressure of the oil separated the marrow and expressed it out of the bone cavity through the larger opening. Occasionally it was necessary to cut each end of the marrow before it could be expressed. The marrow from the epiphyses was removed with a sharp curet and packed first with soft bone wax followed by strips of gauze saturated with wax. The marrow cavity was then cleaned with a pipe cleaner and flushed out with saline (fig. 1). The animal of choice is the rabbit. It is the largest of the animals with a tubular marrow and lends itself for hematologic studies. The animals were anesthetized with pentobarbital sodium. The hair was removed with a depilatory preparation and the leg was wrapped with cotton saturated with an antiseptic. It is essential that the surgical procedure be carried out under strict aseptic precautions.

Forty marrows were extirpated in 30 rabbits in this study. The age of the animals ranged from 3 to 12

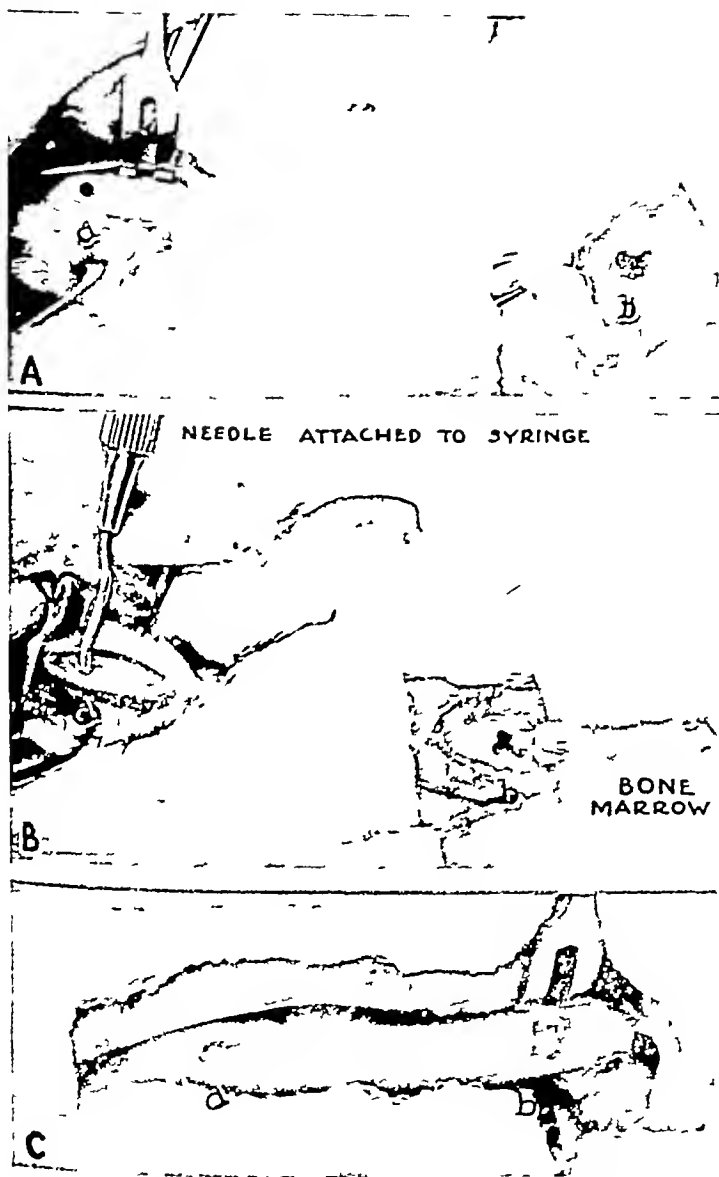


FIG. 1—REMOVAL OF MARROW FROM A LONG BONE OF A LIVING RABBIT

Holes are drilled in the bone with a Rask nail drill. At one end of the bone a single hole is made (a in A). At the opposite end four holes are made and the central bone spicule is removed leaving a large opening (b in A). A flexible silver cannula is inserted into the single hole (a in B) and with a syringe containing oil or water the marrow is expressed through the larger opening (b in B). Both openings are then sealed with bone wax (a and b in C).

months. The animals were given 1 cc. of a mixture of 5 parts of benzene to 1 part of olive oil subcutaneously one to two times daily. The number of injections was varied for each animal (see table 1). Peripheral leukocyte counts were done one or more times daily. The animals were killed at intervals of 3 to 81 days after extirpation of marrow. Studies were made of the comparative changes of the regenerated marrow after fixation in formaldehyde or Bouin's solutions and staining with hematoxylin-eosin or Giemsa preparations.

## RESULTS

### *Regeneration of Normal Marrow*

For a clearer understanding of the comparative changes, the steps in the regeneration of normal marrow are restated. The earliest significant manifestation, which appears in about nine days, is a sprouting of sheets of primitive reticular cells and bone trabeculae from the endosteum. The next step is the formation of fat cells. This process takes place probably by a coalescence of two or more primitive reticular cells after their cytoplasm is replaced by lipids. Fat cells continue to form for sixty days, but their formation is most active and profuse in the first twenty days after extirpation. Islands of myeloid tissue begin to appear in about nine days and increase progressively in number. Regeneration does not proceed uniformly throughout the bone marrow. In sixty days, most of the marrow has returned to a normal number and distribution of myeloid tissue (fig. 2).

### *Regeneration of Marrow in Benzene Intoxication*

The quantity of benzene and the number of injections were varied. Some animals received relatively little of the chemical over a period of a few or many days. Other rabbits were injected almost daily and received a total large quantity of benzene (see table 1). There was a distinct correlation between the degree of intoxication and the appearance of the bone marrow. In severe poisoning, regeneration did not proceed further than the stage of sprouting of primitive reticular cells. There was some attempt to form fat cells, but they were few and atrophic or rudimentary. Whenever an occasional fat cell did develop, it was followed first by proliferation of a few megakaryocytes and then by an infrequent small focus of erythroblasts.

Even after a period of eighty-one days, those animals which received benzene continuously showed a state of marrow response comparable only to the first phase of normal marrow regeneration. Granulocytes did not make their appearance unless a considerable number of fat cells developed and not until both megakaryocytes and cells of the erythrocytic series were present in moderate numbers. A decreasing degree of intoxication was associated with formation of fat cells and myeloid activity. With a relatively small quantity of benzene, fat cells and myeloid tissue was in considerable evidence in twenty-one days after extirpation of the marrow. When benzene administration was stopped, the marrow in the extirpated bone proceeded to develop fat cells, whereas in the intact contralateral marrow, myeloid hemopoiesis would set in.

The significant changes in these experiments consist in the inability of the marrow to regenerate past the primitive reticular cells and the apparent dependence of myeloid activity upon presence of fat cells.

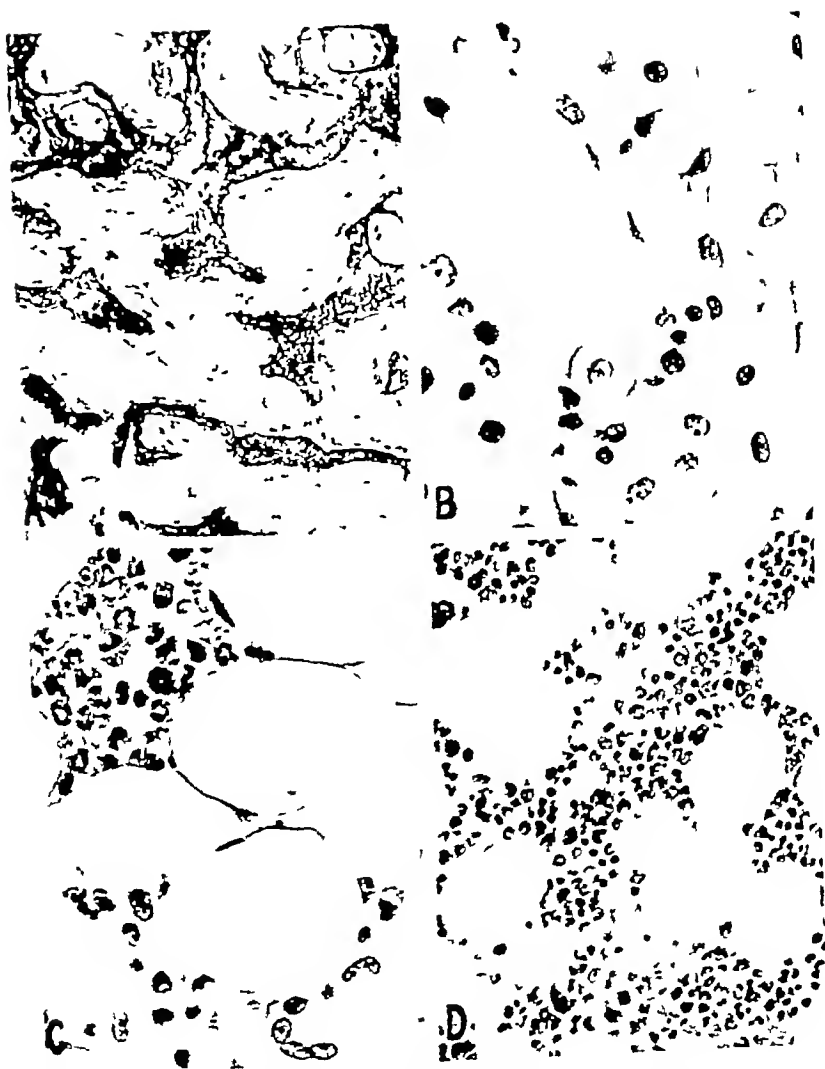


FIG. 2.—A B AND C REGENERATION OF EXTIRPATED MARROW IN NORMAL RABBITS

- A 9 days after extirpation Bone trabeculae and sheets of primitive reticular cells apparently derived from the endosteal layer of bone and from the trabeculae  $\times 100$
- B 20 days after extirpation Formation of fat cells Intermediate forms of primitive reticular cells and erythroblasts are in the field  $\times 720$
- C 30 days after extirpation Islands of myeloid tissue composed of granulocytes and erythroblasts An occasional area of primitive reticular cells  $\times 720$
- D Normal active marrow of a rabbit for comparison  $\times 540$

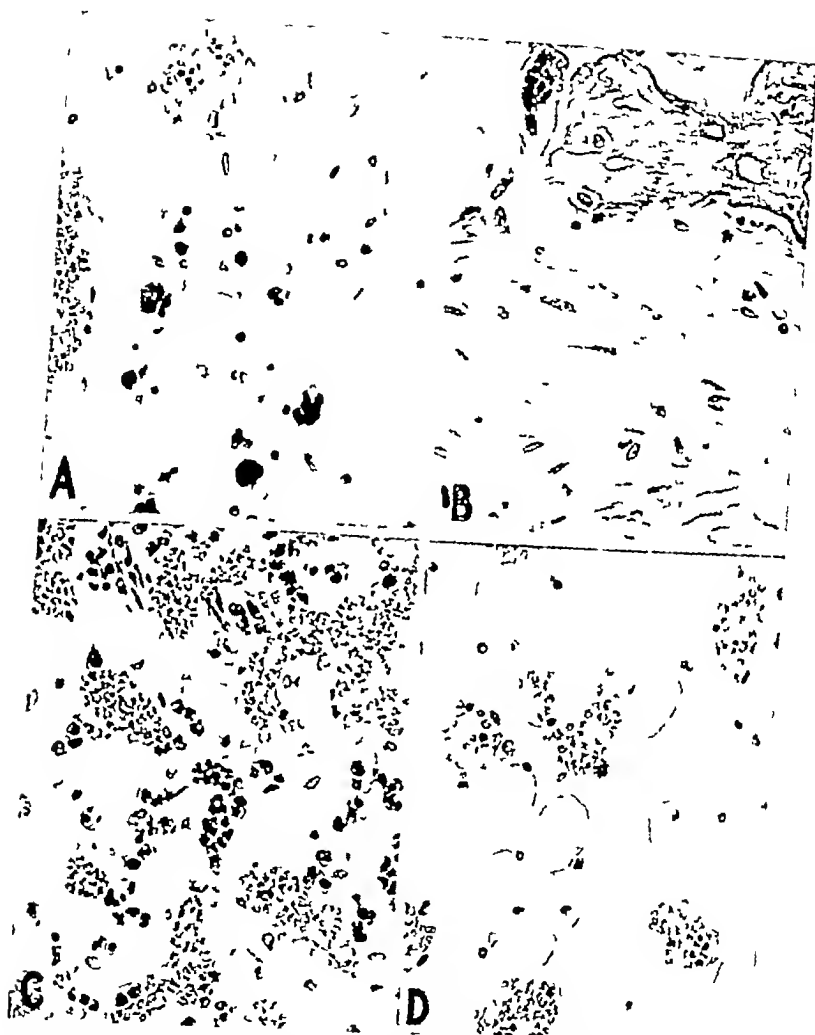


FIG. 3—A AND B REGENERATION OF EXTIRPATED MARROW IN SEVERE BENZENE INTOXICATION

A 54 days after extirpation There are indistinct sheets of primitive reticular cells rudimentary fat cells and megakaryoblasts  $\times 720$

B 81 days after extirpation There are sheets of primitive reticular cells bone trabeculae and rudimentary fat cells but no myeloid activity  $\times 720$

C Intact marrow of same animal as in A There are atrophied fat cells and erythroblastic activity  $\times 540$

D Intact marrow of same animal as in B The fat cells are atrophic There is no myeloid activity  $\times 540$

*Effects of Benzene Intoxication on Intact Bone Marrow*

The intact bone marrow in benzene intoxication will be described only in so far as it helps to clarify the picture of regeneration. Whenever possible, comparisons were made with marrow from controlateral extirpated bones. Marrow from

TABLE 1 — *Relationship of Degree of Benzene Intoxication to Bone Marrow Regeneration*

Period of marrow regeneration	Extent of benzene administration	Range of WBC per cu. mm. of blood during benzene administration	State of the regenerated bone marrow in benzene intoxication
days			
14	9 cc. of benzene for 6 days	1800 to 422	No myeloid cells few fat cells, sheets of primitive reticular cells and bone trabeculae
19	21 cc. of benzene for 11 days	2600 to 420	No myeloid cells no fat cells sheets of primitive reticular cells and bone trabeculae
21	10 cc. of benzene for 7 days	5900 to 2120	Considerable myeloid activity many fat cells and an occasional sheet of primitive reticular cells
30	34 cc. of benzene for 19 days	8900 to 1300	Few areas of myeloid regeneration largely erythroblastic few fat cells extensive sheets of primitive reticular cells and bone trabeculae
32	12 cc. of benzene for 7 days	8160 to 2100	Considerable myeloid activity many fat cells, few areas of primitive reticular cells
30	46 cc. of benzene for 30 days	5700 to 1000	No myeloid regeneration infrequent rudimentary fat cell, extensive sheets of primitive reticular cells and bone trabeculae
33	30 cc. of benzene for 15 days No benzene for 10 days prior to extirpation 5 days during experiment and 5 days before death	6600 to 1086	No myeloid cells few rudimentary fat cells extensive sheets of primitive reticular cells and bone trabeculae
54	93 cc. of benzene for 51 days	7900 to 1850	An occasional megakaryoblast and erythroblast small number of poorly formed fat cells sheets of primitive reticular cells and bone trabeculae
81	157 cc. of benzene for 70 days	9950 to 3100	Few areas of megakaryocytes, few rudimentary fat cells sheets of primitive reticular cells and bone trabeculae

the humerus, femur, radius, ulna and occasionally the ribs was studied. Sheets of primitive reticular cells were not found in any of the marrow even after eighty-one days of benzene administration. The fat cells remained intact in most instances up to fifty-four days. They became atrophic and the nuclei migrated from the periphery to the center of the fat cell in eighty-one days. In some instances of severe in-

## METHOD

We have used the following method to obtain marrow from various small animals (rabbit, guinea pig, mouse and chicken). From small animals such as 8 day old rabbits, guinea pigs and mice, only a few drops of marrow are obtained. From the chicken and from older rabbits (2½ to 8 pounds), 0.5 to 1 c.c. of fluid may be aspirated. At present we are attempting to establish normal myelograms for rabbits of various ages, as a result the majority of our aspirations have been done on rabbits.

The rabbit, under ether anesthesia, is placed on its back, and its legs are secured in the outstretched position. The site of puncture (fig. 1) is the superior medial surface of the tibia inferior to the medial condyle and medial to the tibial tuberosity. This surface is triangular in shape and can be palpated with ease even in extremely small animals. The superficial hair is removed. In the remainder of the procedure, reasonable and adequate precautions with regard to sterile technic are taken. (The needle is allowed to remain in zephiran chloride for several minutes and the operator keeps his fingers moist with the same solution.) We have used the Klima Rosseger needle\* as a biopsy needle but a shortened lumbar puncture needle with a tightly fitting stylet would probably be adequate. A 15 gage needle is preferable for the

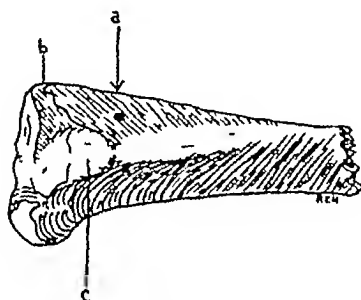


FIG. 1.—ANTERIOR VIEW OF UPPER PORTION OF RIGHT TIBIA OF RABBIT. a Arrow points to site of puncture. b Medial condyle. c Tuberosity.

larger animals. The skin and subcutaneous tissue at the site of puncture are extremely thin. When minimal pressure is applied, the tip of the needle penetrates the superficial tissues and pierces the periosteum. When the periosteum has been penetrated the needle is pushed perpendicularly through the cortex of the tibia until the sudden give sensation indicative of penetration into the medullary cavity is felt. This sudden decrease in resistance to pressure is experienced when attempting to penetrate the cortical bone of any of the adult animals. In young animals, the give sensation is minimal and one may have to judge the depth of penetration by the presence of fat droplets on the stylet. When the marrow cavity has been penetrated the stylet is removed and a 20 cc. syringe is attached. Rapid, strong suction (negative pressure of 10 to 20 cc.) is employed. This usually is followed by the swelling up of marrow into the syringe. Occasionally it is necessary to apply suction more than once in order to obtain fluid, for the marrow may be extremely viscous. Varying amounts of fluid can be obtained, 0.5 cc. is a convenient amount. The needle is removed from the bone and pressure is applied to the wound. Bleeding subsides readily, and no other treatment is required.

The remainder of the method corresponds to that used in the preparation of human sternal marrow for study.<sup>12</sup> The fluid is immediately transferred to a paraffin lined vial containing a minute amount of heparin.† After it has been thoroughly agitated the heparinized marrow is poured out on a clean glass plate. Grossly visible particles of marrow are usually present. The fluid and about half of the particles are transferred to a Wintrobe hematocrit tube by means of a chemically clean capillary pipet. The remaining particles are prepared for microscopic examination by the following method.

\* Made by V. Mueller and Company, Chicago, Illinois.

† Lot 152, Hyoscoo, Westcott and Dunning, Baltimore, Md.

Fix particles in Zenker's fluid—30 minutes to 1 hour

In the remainder of the procedure, remove the fluids from the small vial by means of a capillary pipet. Do not attempt to remove the particles. The timing varies with the size of the particles. A suggested timing is several changes of distilled water—30 minutes 30% alcohol—30 minutes 50% alcohol—1 hour or longer 70% alcohol—1 hour or longer 95% alcohol—10 minutes 100% alcohol (1)—5 minutes 100% alcohol (2)—5 minutes (Add about an equal volume of xylol to this last alcohol almost immediately.) xylol (1)—10 minutes xylol (2)—10 minutes

Remove xylol pour paraffin (M.P. 54°) in vial and allow tissue to become infiltrated in oven for 30 to 45 minutes. Remove paraffin with heated capillary pipet add fresh paraffin and leave in oven for 30 to 45 minutes. Do not leave tissues in oven over 1½ hours.

Remove particles from paraffin by means of a heated capillary pipet. Place tip of pipet at bottom of paraffin-filled boat and force particles out of pipet. The particles should be made to aggregate in a relatively compact mass near the bottom of the boat. Let paraffin harden, pare blocks cut at 5 micra and mount. Stain as desired. Any of the special blood stains can be used following Zenker's fixation. During the staining procedure remove the precipitated mercury. Immediately before staining immerse slides in dilute alcoholic iodine. When the tissue is yellow place slides in a 5 per cent aqueous solution of sodium thiosulphate. Leave slides in thiosulphate until yellow color has faded. Wash in distilled water and continue staining procedure.

The fluid portion is centrifuged at 2500 r.p.m. for eight minutes and readings corresponding to the height of the various strata are taken from the Wintrobe tube. Four main layers (fat plasma myeloid-erythroid, and erythrocytes) are present. Sometimes immediately below the fat layer a layer which consists of a mixture of fat perivascular cells and nucleated marrow cells is found. (These layers give a rough idea of the cellularity of the marrow but sections provide more accurate information in this respect. One of the main advantages of centrifugation is the concentration of nucleated marrow cells to the myeloid-erythroid layer.) The fat and mixed layer are removed and discarded. With a second pipet the myeloid-erythroid layer and a small amount of plasma are transferred to a paraffin lined watch glass. Smears are made from this mixture. The smears are dried rapidly by whipping them through the air or by means of a fan. The smears may be stained with any of the common blood stains. Wright's or the May-Grunwald Giemsa stains are excellent.

Smears made in this way surpass any we have obtained in previous studies of the bone marrow of animals. The smears show isolated undamaged cells in great numbers and are as good for morphologic studies as are those made from human sternal or iliac marrow. Also, as many as thirty cellular smears have been made from a single sample, this has proved valuable for teaching purposes. It has been possible to obtain particulate marrow for sections from almost every rabbit and chicken biopsy.

The procedure described is, of course, not necessary to the study of qualitative changes in the marrow cells. If one withdraws only a few drops of marrow, there is little dilution with sinusoidal blood, and relatively cellular direct smears can be made.

The ease with which marrow can be obtained seems to depend upon the size of the animal and upon the contour of the tibia. Obtaining marrow from the tibia of the guinea pig is reasonably difficult. An 18 gage needle was used, and it was necessary to penetrate at an angle which allowed the needle to be directed toward the shaft of the bone to avoid penetration of the lateral surface of the tibia. In the mouse, the problem is even greater. A short 22 gage needle with an extremely short bevel can be pushed through the cortex of the tibia without too much difficulty. We found it simpler to clear the needle with its stylet after penetrating the cavity rather than attempting to penetrate the bone with the stylet in place.



The needle should be directed toward the shaft of the bone. Only a few drops of marrow were obtained from the guinea pig and the mouse.

No marrow could be aspirated from the tibia or femur of rats. The tibia of the rat has no easily palpable triangular surface. The anterior tibial crest is prominent, and the lateral and medial surfaces of the tibia are in close apposition. When the pressure required for penetration of the cortex of the medial surface was applied, the lateral surface was also penetrated.

The method was not used on dogs, cats, or fowl other than the chicken. Both dogs and cats have triangular surfaces on the medial aspects of their tibias. Except in large dogs, the bone could probably be penetrated with manual pressure.

When 0.5 to 1.0 cc. of fluid are aspirated from the tibia of an adult rabbit, approximately two-thirds of the red marrow in the upper extremity of the bone is evacuated. In one rabbit, aspiration was repeated on days 7, 14, and 30. Only a small amount of fluid was obtained on days 7 and 14, and the percentage of marrow elements was small. One month after the initial biopsy, the fluid was reasonably abundant, but the percentage of immature cells was lower than that in the original marrow. Although repeated biopsies are possible, our method involves aspiration of a large amount of marrow. Biopsies of the same tibia would probably not yield comparable specimens until two months had elapsed.<sup>11</sup>

Aspirations of iliac marrow have been done on rabbits and mice. Approximately 0.5 cc. of fluid can be aspirated from the ilium of the rabbit and treated in the same manner as that obtained from the tibia. Only a few drops of marrow can be aspirated from the ilium of the mouse.

### SUMMARY

1. Methods of aspirating tibial bone marrow from living laboratory animals (rabbit, guinea pig, mouse, and chicken) have been described. No method of aspirating marrow from living mice has been encountered in the literature.

2. The method would probably prove useful in obtaining marrow from the tibia of any small laboratory animal which has a flattened triangular area on the superior medial surface of the tibia.

3. In larger animals (rabbit and chicken), large amounts of marrow can be aspirated. Both smears and sections can be made.

4. The present method, if used in combination with the similar method of aspirating marrow from the ilium, will afford four different sites of aspiration. This should make possible the study of progressive changes in the marrow.

### REFERENCES

- <sup>1</sup> ALEXANDROFF, A. F. Die Morphologie des Sternumpunktiates von Hunden. *Fol. Haemat.* 41: 415-434, 1930.
- <sup>2</sup> BASILE, C. Alcune osservazioni sulla presenza di Leishmanie nei cani. *Atti della Reale Accademia Dei Lincei*. Series 5, 19: 158-160, 1910.
- <sup>3</sup> ERF, L. A. A simple practical and sterile technique for obtaining bone marrow from animals by puncture. *J. Lab. & Clin. Med.* 22: 1080-1082, 1937.
- <sup>4</sup> HO, E. A., CHU, H. J., AND YUAN, I. C. A simple method for obtaining bone marrow from dog. *Chinese M. J.* 38: 679-681, 1940.

- <sup>6</sup> KOLOUCH F A study of the bone marrow plasma cell of mammals with special reference to its origin in the rabbit under normal and experimental conditions Thesis, Univ of Minnesota, 1938 pp 1-84
- <sup>8</sup> MORRIS L. M., AND FALCONER E. H. Intravital bone marrow studies Arch Int Med 30 485-489, 1922.
- <sup>7</sup> MOURIQUAND G., RÉVOL L., AND EDRL, V. Le myélogramme par ponction fémorale chez le cobaye normal Compt rend Soc. de biol 138 250-251, 1944
- <sup>9</sup> NELSON E. Personal communication
- <sup>9</sup> STARKOFF O. Metodo per la biopsia del midollo osseo nelle ricerche sperimentali Annali d Igiene 7 313-317, 1942.
- <sup>10</sup> STEINBERG B. AND MARTIN, R. A. Removal of bone marrow in living animals Proc Soc Exper Biol & Med 61 428-429 1946
- <sup>11</sup> STEINBERG, B., AND HUFFORD V. Development of bone marrow in adult animals Arch Path 43 117-126, 1947
- <sup>12</sup> SUAREZ, R. M. DIAZ RIVERA, R. S., AND HERNANDEZ MORALES F. Aspirated bone marrow studies in normal Macacus rhesus monkeys Amer J M Sc 285 581-586 1943
- <sup>13</sup> SUNDBERG R. D. Sternal aspiration Staff Meet Bull Hosp Univ Minnesota 17 (26) 389-404, 1946
- <sup>14</sup> VOGEL M. T. A simple method for obtaining smears of the bone marrow of the albino rat Tech Bull Reg Med Tech 7 189-190 1946
- <sup>15</sup> WOLFF A. Ueber eine Methode zur Untersuchung des lebenden Knochenmarks von Thieren und über das Bewegungsvermögen der Myelocyten Deutsch Med Wehnschr 29 (10) 165-167, 1903

# THE OCCURRENCE OF THE PERIODIC ACID-SCHIFF REACTION IN VARIOUS NORMAL CELLS OF BLOOD AND CONNECTIVE TISSUE

By GEORGE B. WISLOCKI, M.D., JACK J. RHEINGOLD, M.D.,  
AND EDWARD W. DEMPSEY, Ph.D.

THE PERIODIC acid-Schiff method stains glycogen, mucus, reticulum and basement membranes, some kinds of elastic tissue, fibrin, and various substances of quite unknown composition (McManus<sup>1</sup>, Lillie et al.<sup>2</sup>, Wislocki and Dempsey<sup>3</sup>). Glycogen may be differentiated from these other substances by the fact that it is soluble in saliva.

In applying recent histochemical methods to hematology, we have observed the staining reactions of a variety of normal blood and connective tissue cells by the periodic acid-Schiff procedure. The present paper gives a detailed account of these observations with interpretations of the findings.

## MATERIAL AND METHODS

The principal tissues used were obtained from man and rhesus monkey. The human material consisted of smears of peripheral blood and bone marrow of normal subjects, as well as pieces of uterine tube, uterine cervix, vermiform appendix and mammary gland obtained from operative specimens. A few smears of patients with chronic lymphatic leukemia were also examined. Peripheral blood was obtained by finger puncture and marrow by aspiration usually from the sternum.

The material from young rhesus monkeys (*Macaca mulatta*) comprised bone marrow, spleen, lymph glands and pieces of connective tissue from the mediastinum, peritoneum and skin.

In addition to these, occasional tissues from rabbit (bone marrow of a young animal), sow (endometrial stroma) and rat (various areas of connective tissue) were utilized.

The blood and bone marrow smears as well as the blocks of tissue were fixed in Rossman's mixture (sat. sol. picric acid in abs. alc., 90 cm.<sup>3</sup> formaldehyde (added just before using) 10 cm.<sup>3</sup>). The blocks were embedded in paraffin and sections were cut at 5  $\mu$ . Both the smears and the deparaffinized sections were stained by the periodic acid-Schiff technique. After this fixation and method of staining, glycogen, some acid mucopolysaccharides, fibrin and other substances are stained red or pink. Glycogen is distinguishable from mucus and other positively reacting substances by the use of control sections exposed to saliva. Control sections were placed in saliva at room temperature for one hour before staining them. The periodic acid-Schiff method was applied according to the directions of McManus<sup>1</sup> in slightly modified form. The smears and deparaffinized sections were treated with a 1 per cent solution of periodic acid for five minutes, followed by Schiff's leukofuchsin reagent for fifteen minutes and subsequent rinsing in sulfurous acid. When a counterstain seemed desirable, light green or hematoxylin was used. The sections were then dehydrated in alcohols, cleared in xylol and mounted in balsam.

Besides the regular use of saliva on control sections for the identification of glycogen, a few sections of rabbit and monkey bone marrows were exposed to malt diastase (Fisher Scientific Co.—Eimer and

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From the Department of Anatomy, Harvard Medical School, Boston, Mass.

This work was done in part under a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council to Dr. George B. Wislocki, Department of Anatomy, Harvard Medical School, and in part under a grant from the U. S. Public Health Service to Dr. William Dameshek, Department of Medicine, Tufts College Medical School, for the study of Mediterranean anemia.

\* The human blood and bone marrow was obtained from the Hematological Laboratory of The Pratt Diagnostic Hospital, Boston, Massachusetts, through the interest and courtesy of Dr. William Dameshek.

Amend) The sections were incubated for one hour at 37 C in a 1 per cent solution of malt diastase buffered with phosphate at pH 6.8. The results obtained with diastase differed in some respects from those following the use of saliva as will be discussed in a subsequent passage.

The connective tissues enumerated above were drawn into the investigation for the purpose of studying mast cells and tissue eosinophils. To identify these two cell types with certainty, sections were prepared from the same regions after the use of other fixatives and stains. For the cross checking of mast cells, blocks of tissue were fixed for twelve hours in a 4 per cent solution of basic lead acetate and the sections were stained for thirty minutes in a  $\frac{1}{2}$  per cent aqueous solution of toluidin blue according to the method of Holmgren and Wilander<sup>4</sup> and Holmgren.<sup>5</sup> By this procedure the granules of the mast cells become brilliantly metachromatic. For the identification of eosinophils in connective tissue or bone marrow, blocks fixed in Zenker's fluid were sectioned and stained in eosin and methylene blue. Following this procedure the eosinophils are readily distinguishable by their red-stained granules. Blood platelets, megakaryocytes and polymorphonuclear neutrophils could be readily identified in the periodic acid-Schiff preparations without resorting to other means for checking them. Basophilic leukocytes were uncommon but readily recognizable in peripheral blood. Lymphocytes and monocytes were investigated in human blood smears and in sections of spleen and lymph glands of the monkey.

#### OBSERVATIONS ON MAST CELLS AND TISSUE EOSINOPHILS

*Mast cells* In the connective tissues of man and rhesus monkey mast cells stained quite intensely following the periodic acid-Schiff procedure (fig. 1a). Stained mast cells were encountered in the stroma of the human mammary gland, uterine tube and cervix and, in the monkey, in the stroma of the skin, mediastinum and retroperitoneal tissue. The granules were quite heavily stained but the cytoplasm was also involved to some degree, giving a certain haziness to the granules. The reaction was not abolished by previous treatment with saliva, a result which indicated that the staining was not due to glycogen.

Mast cells encountered in the mucosa of sows' uteri also stained deeply by the periodic acid-Schiff procedure, but the granules were less distinctly differentiated than in the mast cells of monkey and man. This staining was not prevented by previous treatment with saliva. On the other hand, the mast cells in the connective tissues of the rat were rarely and, at best, faintly stained.

In contrast to these species differences, the mast cells of all of these animals exhibited uniform and intense metachromasia of their granules following staining with toluidin blue. Instead of being hazy, the metachromatic reaction was sharply confined to the granules.

*Tissue eosinophils* These cells were found by chance in great abundance in the mucosa of a human vermiform appendix. The eosinophils were easily identified by virtue of their brilliant red granules in sections stained with eosin and methylene blue. By the periodic acid-Schiff technic these same cells exhibited a diffuse reddish staining involving both granules and cytoplasm. This staining was not influenced by treatment with saliva and consequently could not be attributed to glycogen.

#### OBSERVATIONS ON CELLS OF BONE MARROW AND PERIPHERAL BLOOD

*Basophilic leukocytes* Basophilic leukocytes were occasionally picked up in human blood smears. Following the periodic acid procedure, they exhibited a number of brilliantly stained, sharply outlined, small red dots located in a pale pink cytoplasm (fig. 1c). In several control smears exposed to saliva, we were unable to identify any basophils, so that the red-stained material may have been glycogen. This appar-

ent finding needs further verification. In the event that basophilic leukocytes contain glycogen, they would appear to differ from mast cells which contain periodic acid-Schiff positive material which is insoluble in saliva.

*Eosinophilic leukocytes* Eosinophilic leukocytes encountered in human blood smears showed a pink to reddish cytoplasm with clear granules. This staining diminished some, but did not disappear entirely after preliminary exposure of the sections to saliva.

Eosinophilic leukocytes in monkey's bone marrow were quite deeply stained, the granules appearing dark red against a paler background. This staining was not prevented by treatment with saliva (fig 1c). These cells stood out most conspicuously in preparations of marrow which had been treated with saliva which removed the similarly stained glycogen from the neutrophilic leukocytes.

The eosinophilic leukocytes of rabbit's bone marrow contained exceptionally large granules which stained a pale red by the periodic acid technique (fig 1d). The staining of these granules was not influenced by previous treatment with saliva.

*Neutrophilic leukocytes and myelocytes* The neutrophilic leukocytes, in smears and sections of blood and bone marrow of all species investigated, reacted strongly with the periodic acid-Schiff reagents (fig 1b). The antecedent neutrophilic metamyelocytes and myelocytes also reacted positively, the amount of reactive substance being minimal in the myelocytes and increasing as the cells mature into leukocytes. The reaction in the neutrophilic series was completely absent after preliminary use of saliva, indicating that glycogen was responsible for it. Although the glycogen seemed to occur in the cytoplasm in granular or punctate form, it did not appear to be actually localized in the neutrophilic granules, for, as in other

FIG. 1

All of the cells illustrated in this plate were fixed in Rossman's mixture (abs. alc. formaldehyde and picric acid) and were stained by the periodic acid-Schiff method. Figures c, h, i, and j were counterstained with hematoxylin. Figures a to d inclusive and figure j were drawn with a  $\times 90$  objective and a  $\times 15$  ocular, whereas figures e to i inclusive were drawn with a  $\times 90$  objective and a  $\times 10$  eyepiece.

- a Mast cells from stroma of human uterine tube stained after exposure of the section to saliva
- b Neutrophilic leukocytes from the bone marrow of a young rhesus monkey
- c Eosinophilic leukocytes from the bone marrow of a young rhesus monkey stained after exposure of the section to saliva
- d Eosinophilic leukocyte from the bone marrow of a young rabbit, stained after exposure of the section to saliva
- e Basophilic leukocyte from smear of human peripheral blood
- f Megakaryocyte from the bone marrow of a young rabbit
- g Megakaryocytes from the bone marrow of a young rhesus monkey. The cell on the right was untreated, whereas the one on the left was drawn from a section which had been exposed to saliva before staining it.
- h Megakaryocyte and blood platelets (lower left) from smears of human bone marrow and peripheral blood
- i Megakaryocyte and blood platelets (lower right) from smears of human bone marrow and peripheral blood stained after exposure to saliva
- j A typical lymphocyte from a case of chronic lymphatic leukemia showing the maximal number of stained cytoplasmic bodies

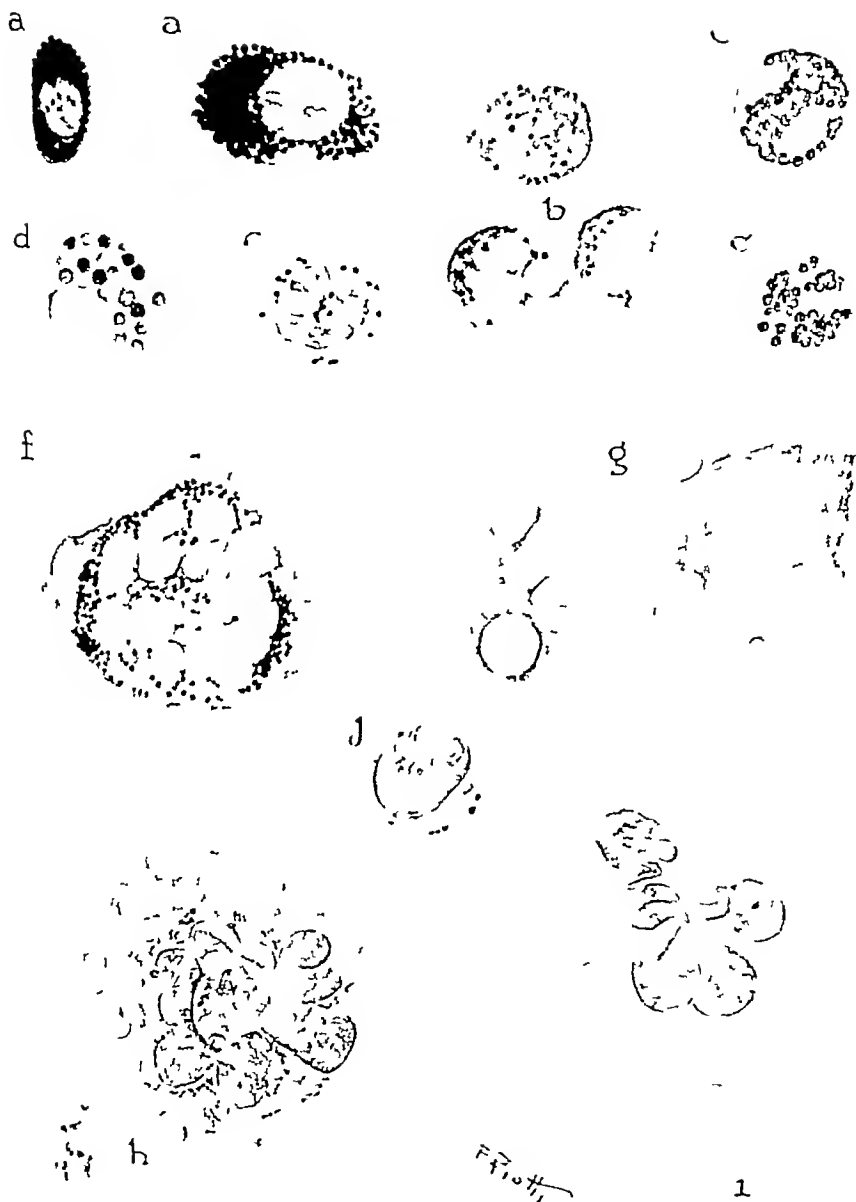


FIG. 1

glycogen-bearing cells, it frequently shifted with fixation to one side of the cell (fig 1b)

*Lymphocytes* The lymphocytes of human peripheral blood were for the most part negative, but about 1 in 10 showed a few deep red Schiff-positive cytoplasmic granules which did not seem to be soluble in saliva. In smears from several patients with chronic lymphatic leukemia, the number of lymphocytes showing these granules was both relatively and absolutely increased. In one case, practically all of the lymphocytes contained from 6 to 12 bright red dots (fig 1j)

The cytoplasm of the lymphocytes observed in stained sections of the spleen and lymph glands of the rhesus monkey was negative

*Monocytes* In smears of human peripheral blood these cells exhibited pale pink, diffuse cytoplasmic staining which did not seem to be influenced by saliva. It was our impression from comparing the staining observed in the various cells of blood and bone marrow that this faint staining of the monocytes was a nonspecific reaction

*Megakaryocytes* In sections of monkey bone marrow these cells exhibited a multitude of indistinct, dustlike, reddish particles located in more faintly stained cytoplasm. This staining was not affected by preliminary treatment with saliva (fig 1g)

In sections of human bone marrow the megakaryocytes exhibited somewhat more intense staining. The diffusely pink cytoplasm contained uneven sized, irregularly scattered, red particles. After treatment with saliva, the red material was no longer visible although the pink background tone survived (figs 1h and i)

In the bone marrow of the rabbit the megakaryocytes stained more intensely than in either monkey or man. Larger red particles filled a good portion of the cells, appearing against a finely punctate reddish background (fig 1f). Treatment with saliva diminished this staining but by no means abolished it.

*Platelets* These were only examined in human peripheral blood. The platelets showed a fine red stippling similar to that seen in the megakaryocytes (fig 1h). This staining failed to occur after exposure to saliva (fig 1i)

### DISCUSSION

*Comparison of saliva and malt diastase* Malt diastase was briefly compared with saliva in reference to its effect on the periodic acid-Schiff reaction and the identification of glycogen. Diastase was tested on several sections of monkey's and rabbit's bone marrow in which neutrophilic leukocytes, neutrophilic myelocytes, eosinophils and megakaryocytes were readily identifiable. Similar to saliva, the use of diastase prevented completely the staining of neutrophilic leukocytes and their myelocytic precursors, but, unlike saliva, it reduced very markedly the staining of both eosinophils and megakaryocytes. These results indicated that saliva and the preparation of malt diastase employed were not completely identical in their action. The latter attacked a wider range of substances than saliva. In connection with other studies we have observed that malt diastase is capable of preventing the staining of basement membranes and reticulum by the periodic acid-Schiff method.

*The nature of the periodic acid-Schiff reaction in blood cells* The action of periodic

acid depends on the oxidation of carbohydrate compounds. As a result, aldehydes are formed and these are revealed by their colored reaction with the leukofuchsin of Schiff's reagent. The reaction produced in some types of blood cells by this technic appears to be due to glycogen, but in other blood cells the saliva-resistant substances which stain must contain other kinds of carbohydrates. In the case of the neutrophilic leukocytes and their myelocytic precursors, the stained substance is undoubtedly glycogen in all species examined. In man the megakaryocytes and platelets also appear to contain glycogen. In the monkey, on the contrary, the megakaryocytes are stained but the substance involved does not seem to be soluble in saliva.

It is well established that the periodic acid-Schiff reaction occurs with a variety of acid mucopolysaccharides (particularly epithelial mucus), and it is probable that the reaction is associated with the carbohydrate fraction of these substances. In mast cells, which possess granules containing an acid mucopolysaccharide, the positive reaction may well be explained in such a way. Species differences exist in the staining of mast cells by the periodic acid-Schiff reagents, in man and monkey their granules stain quite intensely, whereas in the rat they are at best very faintly differentiated. This variability suggests species differences in the availability of the carbohydrate radicals. In this connection it is of interest to note that the intense metachromatic staining of the mast cell granules with toluidin blue shows no such species variability. However, metachromatic staining depends upon the presence of sulphate groups rather than upon the carbohydrate moieties of mucopolysaccharides.

Concerning basophilic leukocytes, there is little that we can say at present. The occasional basophils, encountered in normal blood smears of human blood, contain numerous small red dots in their cytoplasm. The fact that we have not identified any similarly stained cells in several smears exposed to saliva suggests that these droplets consist of glycogen. Yet, these findings seem too few to establish this point definitely. If the above result proves to be consistent, it would indicate a difference in the histochemical composition of mast cells and basophilic leukocytes.

In a previous investigation of the blood cells of the rhesus monkey by the Bauer-Feulgen method, Wislocki and Dempsey<sup>6</sup> observed that only the polymorphonuclear neutrophils and their metamyelocyte precursors gave a positive reaction, and this staining was shown to be due to glycogen. Subsequently, Rheingold and Wislocki<sup>7</sup> described the megakaryocytes of human marrow as giving a faint Bauer-Feulgen reaction in contrast to the negative megakaryocytes of the rhesus monkey. Comparison of these previous findings with the present ones indicates that the Bauer-Feulgen technic, as we have carried it out, is not as sensitive as the periodic acid-Schiff reaction. Regardless, however, of the fact that the two methods have not been quantitatively alike, as we have used them, they have corroborated one another in indicating that there are histochemical differences between the megakaryocytes of man and rhesus monkey.

The reaction in the several types of eosinophils does not appear to be due to glycogen. Nor can it be possibly ascribed to an acid mucopolysaccharide when one



considers the fact that the cytoplasm of these cells is alkaline in nature. It is conceivable that it might be attributable to the presence of a neutral mucopolysaccharide. Noteworthy also is the observation that, whereas in the eosinophils of the bone marrow of rabbit and monkey it is principally the granules which are stained, in the eosinophils of human peripheral blood it is the cytoplasmic ground substance which is chiefly colored.

### SUMMARY

An account is given of the periodic acid-Schiff reaction in the cytoplasm of various normal cells of blood and connective tissue of man, rhesus monkey and rabbit. Saliva treated control sections were used to distinguish glycogen from other reactive substances. The effects of malt diastase were compared briefly with those of saliva. The results of the present study may be summarized as follows (table 1).

TABLE 1—*Brief characterization of the cytoplasmic staining of various cells of blood and connective tissue by the periodic acid Schiff method with saliva controls*

Glyc = Positive reaction due to glycogen. Pos. = Positive reaction presumably due to other carbohydrates. Ft = Faint and questionable reaction, Neg = No reaction. \* = Not crucially tested with saliva. † = Normally positive in about 1 cell out of 10. Blank spaces indicate that no observations were made.

Cell	Man	Monkey	Rabbit
Neutroph leuk.	Glyc.	Glyc.	Glyc.
Neutroph myel.	Glyc.	Glyc.	Glyc.
Eosinoph leuk.	Pos.	Pos.	Pos.
Basoph leuk.	*Pos.		
Lymphocytes	†Pos.	Neg.	
Monocytes	Ft.		
Megakaryocytes	Glyc.	Pos.	Pos.
Blood platelets	Glyc.		
Tissue eosinoph.	Pos.		
Mast cells	Pos.	Pos.	

### REFERENCES

- McMANUS J. F. A. Histological demonstration of mucin after periodic acid. *Nature* 158: 202, 1946.
- LILLIE R. D., LASKEY A., GRECO, J. AND JACQUIER H. Studies on the preservation and histologic demonstration of glycogen. *Bull. Internat. Med. Museums* 27: 23, 1947.
- WISLOCKI, G. B., AND DEMPSEY, E. W. The chemical histology of the human placenta and decidua with reference to mucoproteins, glycogen, lipids and acid phosphatase. *Am. J. Anat.* 43: 1, 1948.
- HOLMGREN H., AND WILANDER O. Beitrag zur Kenntnis der Chemie und Funktion der Ehrlichschen Mastzellen. *Zeitsch. f. mikr. anat. Forsch.* 42: 279, 1937.
- HOLMGREN H. Studien über Verbreitung und Bedeutung der chromotropen Substanz. *Zeitsch. f. mikr. anat. Forsch.* 47: 489, 1940.
- WISLOCKI, G. B., AND DEMPSEY, E. W. Observations on the chemical cytology of normal blood and hemopoietic tissues. *Anat. Rec.* 96: 249, 1946.
- RHEINOOLD J. J., AND WISLOCKI, G. B. Histochemical methods applied to hematology. *Blood* 3: 641, 1948.

## GLYCOGEN IN HUMAN BLOOD CELLS

By ROBERT P GIBB, M D \* AND ROBERT E STOWELL, M D

NEW IMPROVED histochemical technics for detecting glycogen in tissues justify reinvestigation of the distribution of glycogen in human blood cells. Histochemical methods permit more precise observations on the localization of glycogen within single cells of different types than are obtained by macrochemical analyses. Most investigators have claimed that glycogen is present in only certain types of white blood cells. The application of improved technics should yield facts permitting a better understanding of the metabolism and functions of normal and abnormal cells. In his recent review of the functions of leukocytes, Rebuck<sup>1</sup> emphasizes the contributions which histochemical studies have already made to the advancement of our knowledge of leukocytic function. Furthermore, histochemical technics should also be explored for possibilities in providing improved methods for diagnosis of disease in which morphologic differences in blood cells are not easily distinguished.

Therefore, the Gomori<sup>2</sup> and the Hotchkiss<sup>3</sup> technics for demonstrating glycogen have been applied to the study of glycogen in normal and abnormal peripheral blood and bone marrow. The results were compared with those obtained by other histochemical and by macrochemical methods in this and other laboratories.

Neukirch<sup>4</sup> in 1910 noted granular material in polymorphonuclear neutrophiles which were stained by iodine and Best's carmine. He attributed these granules either to glycogen or some closely related carbohydrate. In platelets a centrally located granule was stained by Best's carmine, but this was not removed by salivary digestion. Iodophilic granules were described in myeloid cells, lymphocytes, platelets, and megakaryocytes by Stahl, Horstmann, and Hilsnitz in 1925.<sup>5</sup> In 1941, histochemical studies of blood glycogen by Mancini and Celani Barry<sup>6</sup> compared the results of the Bauer-Feulgen technic with the iodine and Best's carmine methods on dried blood films. Glycogen-positive granules were described in cells of the myeloid series. Polymorphonuclear neutrophiles produced the most intense reactions. Altmann-Gersh freezing fixation revealed larger quantities and more regular distribution of glycogen than did chemical fixation.<sup>7</sup> Lymphocytes and monocytes in all blood films studied in man and in corresponding cells of other animals were not observed to contain glycogen. Using paraffin sections of peripheral blood and hemopoietic tissue stained with Bauer-Feulgen and Mitchel and Wislocki's<sup>8</sup> ammoniacal silver technics, Wislocki and Dempsey<sup>9</sup> in 1946 observed glycogen in neutrophilic leukocytes but not in other blood cells, megakaryocytes, or blood platelets. In these investigations absence of the reactions in control films or sections exposed to salivary digestion prior to staining was proof that glycogen was the substance demonstrated.

From the Department of Pathology, Washington University School of Medicine, St. Louis, Mo.

This investigation was aided by a grant from the American Cancer Society on the recommendation of the Committee on Growth of the National Research Council.

\* Jackson Johnson Scholar, Washington University School of Medicine.

## MATERIALS AND METHODS

Peripheral blood and bone marrow films were prepared on chemically clean cover glasses and were air-dried and stored for periods of a few days to four months before staining. Storage for periods longer than four months usually resulted in a diffuse precipitation on the films during staining but did not significantly alter cellular glycogen content. Just prior to staining the films were fixed in absolute alcohol from one to two minutes, dipped in 95 per cent ethyl alcohol, and placed in 50 per cent ethyl alcohol for two or three minutes. Coating films with celloidin was found to be unnecessary.

The first method used to stain glycogen was the chromic acid-silver methenamine technique described by Gomori.<sup>2</sup> The oxidation of the glycogen with liberation of free aldehyde groups produced a localizing black deposit of reduced metallic silver. Aqueous safranin was used as counterstain.

The second technique employed was developed by Hotchkiss<sup>3</sup> and differs from that described by McManus<sup>10</sup> and by Lillie<sup>11, 12</sup> in that a periodic acid solution buffered with sodium acetate was used to liberate the free aldehyde groups from the glycogen molecule. The films were counterstained with a basic dye using either methylene blue, light green, or toluidin blue. Glycogen stained reddish purple.

A few films were treated with the Bauer Feulgen<sup>13</sup> technique for comparison of results. Control films exposed forty-five minutes to salivary digestion were prepared with each group of films and each technique.

Peripheral blood films were examined on 9 normal subjects. Morphologically normal peripheral blood (12\*) and bone marrow films (11) were studied from patients with carcinoma (5), Laennec's cirrhosis (3), thyrotoxicosis (3), myxedema, nutritional deficiencies, syphilis, Hodgkin's disease, Addison's disease, and hepatolenticular degeneration (Wilson's disease). Peripheral blood films were also examined from patients with a variety of blood conditions including lymphoid leukemia (9), leukocytosis (8), myeloid leukemia (3), infectious mononucleosis (3), mycosis fungoides with eosinophilia (2), aplastic anemia (2), hypoglycemic shock (2), leukopenia (2), and one case each of monoblastic leukemia, monocytic leukemia, polycythemia, thrombocytopenic purpura, hemophilia, pertussis with lymphocytosis, hypoplastic anemia, and hemolytic anemia. Bone marrow films were examined from patients with polycythemia (7), lymphoid leukemia (5), pernicious anemia (5), hypoplastic anemia (4), myeloid leukemia (2), multiple myeloma (2), and one case each of monoblastic leukemia, reticulum cell sarcoma, thrombocytopenic purpura, agnogenic myeloid metaplasia, leukemoid blood picture, mycosis fungoides with eosinophilia, strongyloidiasis with secondary anemia, and hemolytic anemia †.

Macrochemical determinations of whole blood were made on the fasting blood of two normal people and 2 patients with lymphatic leukemia. The technique employed was essentially that described by Good, Kramer, and Somogyi<sup>14</sup> with slight modifications. Following hydrolysis, glucose was determined by the method described by Nelson<sup>15</sup> using the Klett-Summerson photoelectric colorimeter. Yeast-fermented controls were also run. Similar glycogen determinations on leukocytes on the buffy coat of centrifuged blood were less satisfactory.

## RESULTS

The silver reduction method of Gomori and periodic acid fuchsin sulfite method of Hotchkiss produced essentially the same results on normal blood films. When present in small amounts, glycogen was more readily observed by the silver method because of the sharp contrast produced by the black precipitate. For this reason, this technique was preferred for the study and photography of the cells. Because of the nonspecific tinting of the films which occurs with the Bauer Feulgen technique, it was difficult to study and evaluate the presence or absence of small amounts of glycogen. Where larger amounts of polysaccharide were present, the results did not differ significantly from those obtained with the Gomori and Hotchkiss techniques. Diffuse tinting of extracellular material was observed with the Hotchkiss

\* The number enclosed in parenthesis is the number of normal subjects or patients studied.

† Appreciation is expressed to Dr. Carl V. Moore, Department of Medicine, Washington University School of Medicine for facilitating the obtaining of the clinical material.

technic only in very thick areas of bone marrow films. Otherwise the reaction was strictly localized in the cellular cytoplasm. Light green or toluidin blue counterstain aided visualization of small quantities of glycogen in both the Bauer-Feulgen and Hotchkiss technics.

*Normal subjects* Using the technics described, erythrocytes, normoblasts, and erythroblasts of normal blood and bone marrow did not reveal detectable amounts of glycogen. Megaloblasts\* were not identified in the normal films. All myeloid cells contained significant amounts of glycogen with the Hotchkiss and Gomori technics. Segmented polymorphonuclear leukocytes were most intensely stained while the glycogen was decreased in the younger cells as exemplified in figures 1 and 2. Not only was there a diminution in the total amount of glycogen corresponding to the decrease in cytoplasm in these younger cells, but there was also a lower glycogen concentration as manifested by a reduced intensity of the histochemical reactions. Individual identification and classification of the younger myeloid elements was not possible, but, by comparison of percentiles of various types and rough morphologic comparison of films of the same marrow stained with Wright's stain, it was possible to conclude that cells as young as the myelocyte. C neutrophils contained moderate amounts of glycogen and younger cells probably contained at least slight amounts. All identifiable myeloid cells contained significantly detectable amounts of glycogen. In the young cells the glycogen was present in a finely granular form evenly distributed throughout the scant cytoplasm. We attempted to evaluate quantitative differences in individual polymorphonuclear neutrophils. After considering cell maturity, cell size, differences of intensity for a given film and for a given area of the film being studied, it was difficult to appreciate significant alterations in the quantities of glycogen in the individual cells of a given age. Using the Gomori technic, differences were noted in the form and distribution of cytoplasmic granules of mature polymorphonuclear neutrophils. The glycogen was usually distributed as an almost solid, dense cytoplasmic conglomeration of granules. These were frequently so numerous that they overlay portions of the nucleus, not uncommonly obscuring most of it. Occasionally the granules were more diffuse in distribution and finer in structure. This produced a gray coloration to the cytoplasm rather than the jet black which was most commonly observed. Dense homogenous appearing clumps of glycogen were occasionally present with the smaller more diffuse distribution of granules. These differences were not apparent by the Hotchkiss or Bauer-Feulgen technics.

Eosinophilic leukocytes were easily identified in the silver stained films by their typical large granules and as is shown in figure 5 were silhouetted in bold relief against a black background of cytoplasmic silver stained glycogen. Eosinophilic myelocytes in the bone marrow likewise contained glycogen, but in reduced amounts as described for neutrophilic myelocytes. These granules were also visualized by the Hotchkiss and Bauer-Feulgen methods, but were not outlined as clearly. Basophilic leukocytes were not identified.

All lymphocytes observed in films stained deeply with the Gomori technic con-

\* Polyploletic terminology as employed by Sabin



Peripheral blood and bone marrow cells stained with Gomori's silver methenamine technique and safranin counterstain. Glycogen stains black.

FIG 1—Lymphocyte, eosinophilic leukocyte, two segmented polymorphonuclear neutrophils and several platelets, normal peripheral blood film ( $\times 500$ ).

FIG 2—Immature myeloid cells, morphologically normal bone marrow ( $\times 1215$ ).

FIG 3—Megakaryocyte with nucleus obscured by glycogen; platelets arising from periphery, normal bone marrow ( $\times 1215$ ).

FIG 4—Three lymphocytes and a platelet containing small amounts of glycogen, lymphoid leukemia bone marrow ( $\times 1215$ ).

FIG 5—Eosinophilic leukocyte shown in Fig 1 revealing eosinophilic granules and extragranular glycogen ( $\times 1215$ ).

FIG 6—Polymorphonuclear leukocyte, myelocyte A and blast cells showing small amounts of cytoplasmic glycogen, peripheral blood of patient with acute myeloid leukemia ( $\times 1215$ ).

FIG 7—Lymphocytes and polymorphonuclear neutrophils showing removal of glycogen by saliva digestion before staining, strongly counterstained, lymphoid leukemia bone marrow. Same marrow as Fig 4 ( $\times 1215$ ).

tained glycogen. The amounts were small and produced a thin black rim of granules around the relatively large nucleus as shown in figure 1. Occasionally the glycogen of the lymphocytes was manifest as a few large granules located in areas where the cytoplasm was greatest in amount. The quantity of glycogen varied directly with the amount of cytoplasm visible. The larger and presumably younger cells contained, therefore, more glycogen. The Hotchkiss method revealed glycogen in almost all lymphocytes. With the Bauer-Feulgen technic glycogen granules were present in a few lymphocytes.

All monocytes contained a moderate amount of glycogen, more diffusely distributed and superimposed over portions of the nucleus and characterized by smaller granules than those of the polymorphonuclear neutrophils. The carbohydrate was demonstrable by all three technics.

The three methods employed revealed glycogen in both megakaryocytes and platelets. In both of these elements the polysaccharide appeared in two forms as can be seen in figures 1, 3, 4, 8 and 9. A finely granular form was diffusely distributed in the cytoplasm and over parts of the nucleus in megakaryocytes and was present in the peripheral portion of the platelets. In the majority of the megakaryocytes deeply staining homogenous appearing clumps of glycogen overlay a large part of the nucleus, and in the platelets a similarly deeply staining centrally located clump was present (fig. 3). Occasionally parts of this homogenous substance in the megakaryocytes stained a deep brown color and blended smoothly into the adjacent black material. The amount varied from a few small granules to a large clump or clumps of glycogen occupying about two-thirds of the cell.

*Myeloid Leukemia* Leukemic myeloid cells did not differ significantly from cells of similar age and size seen in normal peripheral blood and bone marrow. The leukocytes observed in the peripheral blood of one patient containing 71 per cent myelocyte A cells and 12 per cent myeloblasts showed significant quantities of glycogen. Figure 6 shows that the glycogen in these young cells was small in amount and uniformly distributed as fine granules in the thin rim of cytoplasm. Other formed elements of the blood did not differ significantly in glycogen content from similar elements in normal blood and bone marrow.

*Lymphoid Leukemia* In adequately stained films leukemic lymphocytes contained glycogen. The small amount was frequently evident as a few cytoplasmic granules as shown in figure 4. These cells vary in the speed of their response to the silver-methenamine reaction. In weakly stained preparations up to one-half of the lymphocytes showed negative reactions. In duplicate films stained for longer periods of time all lymphocytes observed contained detectable granules. Myeloid cells, monocytes, and platelets when present contained glycogen granules in amounts which did not differ significantly from those observed in the normal.

*Monoblastic Leukemia and Monocytic Leukemia* Leukemic monocytes contained a moderate amount of glycogen similar in distribution to that seen in monocytes in

FIG. 8.—Multiple myeloma cells with moderate amounts of cytoplasmic glycogen and a platelet with a small amount of glycogen. bone marrow of patient with multiple myeloma ( $\times 1,215$ )

FIG. 9.—Segmented polymorphonuclear neutrophils showing dense concentrations of glycogen and a platelet with a small amount of glycogen. bone marrow of patient with polycythemia ( $\times 1,215$ )

normal blood films Monoblasts in the bone marrow and peripheral blood of a patient with monoblastic leukemia contained a few granules of glycogen in the cytoplasm These monoblasts constituted 99 and 94 per cent of the leukocytes in the bone marrow and peripheral blood films respectively

*Multiple Myeloma* Myeloma cells were identified in the bone marrow of two patients with this disease One film contained 71 per cent and the other 18 per cent myeloma cells As shown in figure 8 the abundant cytoplasm of these cells contained glycogen in a moderately fine granular form These patients had not been treated with stilbamidine and cytoplasmic inclusion bodies of the type described by Snapper<sup>16</sup> were not present in films stained with Wright's stain Glycogen in leukocytes in films from patients with multiple myeloma did not differ significantly from that observed in similar cells of normal bone marrow

*Infectious Mononucleosis* With the stains employed, it was not always possible to differentiate with certainty between the cells of infectious mononucleosis, monocytes, and large lymphocytes Glycogen was not visualized in large cells morphologically similar to the characteristic cell of infectious mononucleosis Other cells similar in appearance but presumed to be large lymphocytes or monocytes contained slight to moderate amounts of glycogen

TABLE 1

Normal Subjects	WBC/mm <sup>3</sup>	Glycogen in whole blood in mg %	Glycogen per million WBC
A1	6,200	6.58	1.06
A2	5,850	7.22	1.24
A3	5,900	5.49	0.93
A4	6,700	6.90	1.01
B1	6,000	4.71	0.79
B2	6,000	7.56	1.21
<hr/>			
Lymphatic Leukemia			
C1	165,000	10.47	0.06
D1	74,850	3.19	0.11

*Polycythemia* Polymorphonuclear cells from patients with polycythemia stain intensely Dense homogenous-appearing cytoplasmic clumps of glycogen similar in appearance to those in the normal were present in large amounts in mature polymorphonuclear leukocytes as shown in figure 9 Platelets and megakaryocytes reacted strongly The quantity of glycogen in lymphocytes and monocytes did not differ significantly from that described in normal cells

*Other conditions* The formed blood elements from the peripheral blood and bone marrow of the sampling of patients with leukocytosis, leukopenia, the anemias, hypoglycemia, diabetes, thyrotoxicosis, myxedema, Addison's disease, cirrhosis, thrombocytopenic purpura, hemophilia, carcinoma, reticulum cell sarcoma, Hodgkin's disease, agnogenic myeloid metaplasia, mycosis fungoides, syphilis, and pertussis contained amounts of glycogen which did not differ appreciably from similar cells in normal films Abnormal cells were not identified in the bone marrow films of reticulum cell sarcoma or carcinoma

Saliva completely removed the glycogen from myeloid, lymphoid, monocytic and plasma cells, and platelets and megakaryocytes as evidenced by the absence of reaction with the histochemical technics employed. Figure 7 illustrates this removal of glycogen in lymphocytes and a segmented polymorphonuclear neutrophil in the bone marrow from a patient with lymphoid leukemia.

The results of macrochemical glycogen analysis of whole blood and buffy coat are summarized in table I. Blood from patients with lymphoid leukemia, C<sub>1</sub> and D<sub>1</sub>, had differential counts of 4, 96, 0 and 2, 97, and 1 per cent polymorphonuclear leukocytes, lymphocytes, and monocytes respectively while the mean percentages for the normals was 72, 18, and 10. Significant glycogen values of 10.47 mg per cent and 3.19 mg per cent were obtained in two cases of lymphoid leukemia. Following yeast fermentation only traces of nonfermentable reducing substances were present. These did not significantly alter the glycogen values obtained.

### DISCUSSION

The histochemical demonstration of greater quantities of glycogen than has been previously reported may be attributed in part to the greater sensitivity of the histochemical methods used in this study. Gomori<sup>2</sup> has adequately discussed the features of his technic which increase the sensitivity over the Bauer-Feulgen and the Mitchel and Wislocki ammoniacal silver technics. The increased sensitivity of the Hotchkiss method may be due to the fuchsin-sulfite which is decolorized and cleared with charcoal to eliminate the diffuse tinting that obscures small quantities of the carbohydrate in the Bauer-Feulgen method. Because of the marked contrast which the black reduced silver produced in blood films, the Gomori technic is superior for the detection of minute quantities of glycogen in blood cells.

The demonstration of greater quantities of glycogen may also be attributed in part to the use of blood films rather than tissues prepared by histological methods. Although glycogen does have a low solubility in alcohol solutions, a small loss is to be expected by washing in the large number of solutions which are required for the preparation of histologic sections. Even though these have been reduced to a minimum in the methods used, it is not improbable that some loss of glycogen still occurs. K. H. Meyer<sup>17</sup> has pointed out that glycogen exists in varying degrees of polymerization and the greater the degree of polymerization the less water soluble is the glycogen. On a theoretic basis the low polymer molecules which are the most readily lost would also be the most difficult to demonstrate histochemically for the concentration of potentially free aldehyde groups would not be as great. These aldehyde groups are located between carbon atoms with free hydroxyl groups and their concentration would be proportional to the number of polymerized glucose radicals. As Hotchkiss<sup>2</sup> emphasizes, low molecular compounds such as simple sugars, hydroxyamino acids with adjacent hydroxyl and amino radicals and substituted inositol compounds can react with the periodic acid reagent, but these are not normally present in fixed preparations. The pentose component of nucleic acids is so substituted that it does not react with periodic acid. Cerebrosides would be expected to react if they were retained in the preparations. Hotchkiss believes that the principal substances which would be expected to show the periodic acid fuchsin sulfite stain in animal tissues are glycogen, mucin, mucoprotein, and presumably hyaluronic acid and chitin. Lillie has shown that acidi-



fied sodium periodate solution will also react with collagen, reticulum and fibrin <sup>11, 12</sup> The use of salivary digestion of control tissues should permit the reasonably definite identification of glycogen with these technics

This discussion has so far ignored the results obtained using iodine stains The chemistry of this reaction is poorly understood and most investigators believe that iodine is usually more non-specific and produces more diffuse tinting than does the Bauer-Feulgen technic Mancini and Celani Barry<sup>6</sup> do not share this opinion and have based their conclusions chiefly on observations employing iodine staining of blood films They did observe greater quantities of glycogen in neutrophilic leukocytes than workers using tissue sections prepared by histologic methods, but were not able to observe the small quantities of glycogen in other blood cells It seems probable that these small quantities of glycogen may be obscured by the diffuse tinting which iodine produces The iodophile granules observed by Stahl, Horstmann, and Hilsnitz<sup>5</sup> in neutrophils, lymphocytes, platelets, and megakaryocytes were most likely glycogen granules Their observations have now been confirmed by three histochemical methods, with absence of staining in respective control sections The present observations do not agree with the findings of Stahl, Horstmann and Hilsnitz that glycogen appears in erythroblasts and increases with progressive maturity of these cells The bronze coloration which iodine produces in erythroid cells parallels the appearance of, and increases with increasing hemoglobin content and might be interpreted more as a reaction with hemoglobin than with glycogen Although glycogen in erythrocytes has been reported by Ellis and Payne<sup>18</sup> using macrochemical methods it has been denied by many other workers (Bridge and Holt,<sup>19</sup> Van Creveld,<sup>20</sup> Wagner<sup>21</sup>)

These observations of glycogen in cells of the myeloid series are in full agreement with the work of Mancini and Celani Barry<sup>6</sup> They do not mention eosinophilic leukocytes specifically, but may have included them in their general term myeloid cells Wislocki and Dempsey<sup>9</sup> reported glycogen in neutrophilic leukocytes but were unable to identify eosinophilic leukocytes with certainty in their preparations Eosinophilic leukocytes were identified in our preparations (fig 4), but not basophilic leukocytes Because myeloid cells which were free from glycogen were not observed in normal peripheral blood and bone marrow films, basophiles, although not specifically identified, may also contain glycogen

Because of the intense reactions occurring in the mature polymorphonuclear neutrophils with all three technics, it was not possible to demonstrate azurophilic or neutrophilic granules in the glycogen preparations The glycogen was present in younger cells than those containing neutrophilic granules in Wright's stained films It was present in larger amounts than might be accounted for if present only in azurophilic granules Granules of glycogen were in all cases larger in size than neutrophilic granules of films stained with Wright's stain The carbohydrate was also present in eosinophilic leukocytes, plasma cells, platelets, and megakaryocytes which do not contain neutrophilic or azurophilic granules Eosinophilic granules were definitely outlined by the cytoplasmic glycogen Therefore, it cannot be concluded that glycogen is a component of neutrophilic, azurophilic or

eosinophilic granules, rather, it is believed that the glycogen is contained in the extra granular cytoplasm.

Wright's postulated origin of platelets from megakaryocytes<sup>22</sup> is further substantiated by the histochemical demonstration of glycogen in two morphologically similar forms in both elements. As visualized in figure 3 the formation of platelets from the periphery of the megakaryocytes is evident in the preparations.

Contrary to the observations of previous workers, moderate amounts of glycogen in monocytes, and small amounts of glycogen in lymphocytes were observed and were demonstrated by three different methods. This glycogen was also removed by salivary digestion. The glycogen of leukemic myeloid monocytic, and lymphoid cells did not differ significantly in amount and distribution from normal cells of the same stage of maturation. It was not possible to demonstrate a histochemical difference in the content of glycogen in leukemic myeloblast and monoblast cells.

The glycogen content of myeloma cells to our knowledge has not been previously reported. It will be of interest to compare the glycogen content of normal plasma cells with the myeloma cells.

The dense homogenous clumps of glycogen which were observed in the polymorphonuclear leukocytes of polycythemia were suggestive of increased glycogen in these cells. Because of the intense reactions which also occur in the normal, it was difficult to interpret the significance of this apparent increase. Platelets and megakaryocytes also reacted strongly. Wagner<sup>22</sup> has reported an increase in the glycogen content of whole blood and in isolated leukocytes in this disease as determined by macrochemical methods.

Large mononuclear cells which did not contain glycogen were observed in blood films of three patients with infectious mononucleosis. These cells could not be differentiated with certainty from monocytes and large lymphocytes, both of which are also increased in the blood of patients with this disease. Monocytes and lymphocytes in the blood of normal subjects and patients with other diseases contain glycogen. These large mononuclear cells were the only leukocytes observed in this study which did not contain glycogen.

The demonstration of glycogen in lymphocytes and platelets does not agree with the results of macrochemical determinations by Wagner.<sup>21, 22</sup> From studies of whole blood and isolated buffy coat leukocytes from normal individuals and from patients with different types of leukemias, he concluded that the granular leukocyte is the only carrier of glycogen in whole blood. He also stated that lymphocytes, blast cells, and platelets do not contain any measurable amounts of glycogen. The experience obtained in the present study with a method of glycogen determinations comparable to that employed by Wagner corroborates his findings, that the values obtained would not indicate a significantly measurable amount of glycogen in lymphocytes. It should be stressed, however, that the wide variations in normal values which he obtained, 1.2 to 16.2 mg per cent in 42 determinations on 28 normal individuals, might indicate a wide range of error in the method. Wagner found 10.9 mg per cent and 14.4 mg per cent whole blood glycogen in two patients with chronic lymphatic leukemia and blood differential counts show-

ing 100 per cent lymphocytes. Although these glycogen values are within the normal range and might be attributed to granular leukocytes present in quantities less than 1 per cent of the differential picture, the slight increase in glycogen could well be correlated with the increased number of lymphocytes. Only a more extensive study of the glycogen content of the blood of patients with lymphatic leukemia would reveal if this is a significant increase. The authors believe that the methods now available for the macrochemical determinations of glycogen are not sufficiently delicate to quantitatively measure this substance in the small amounts which are demonstrable histochemically. It is evident that the number of lymphocytes (fig. 4), platelets (figs. 1 and 3), or blast cells (fig. 6) which would contain the equivalent of the amount of glycogen present in one polymorphonuclear leukocyte is very large, possibly a hundred or more.

### CONCLUSIONS

Three different histochemical methods were applied to the study of glycogen in normal and abnormal peripheral blood and bone marrow films. The techniques employed were the chromic acid-silver-methanamine procedure of Gomori, the periodic acid-fuchsin-sulfurous acid technic of Hotchkiss and the Bauer-Feulgen stain. Control sections were treated with saliva to remove the glycogen.

Large amounts of glycogen were demonstrated in the cytoplasm of polymorphonuclear, metamyelocytic, and myelocytic neutrophilic leukocytes and in the extra granular cytoplasm of eosinophilic leukocytes in films from normal individuals. Megakaryocytes and the cytoplasm of monocytes contained moderate amounts of glycogen, and platelets and the cytoplasm of lymphocytes smaller amounts.

Examination of peripheral blood and bone marrow of patients with a variety of hematologic, metabolic, and infectious diseases failed to reveal significant differences in glycogen content from the normal with the possible exception of polycythemia in which a suggestive increase in cellular glycogen was observed in polymorphonuclear leukocytes, platelets, and megakaryocytes. A moderate amount of glycogen was observed in multiple myeloma cells. Large cells in patients with infectious mononucleosis did not stain for glycogen.

### REFERENCES

- <sup>1</sup> REBUCK J. W. Functions of leucocytes. *Am J Clin Path* 17: 614, 1947.
- <sup>2</sup> GOMORI G. A new histochemical test for glycogen and mucin. *Am J Clin Path* 14: 177 (tech. Sec.) 1946.
- <sup>3</sup> HOTCHKISS R. D. A microchemical reaction resulting in the staining of polysaccharide structures in fixed tissue preparations. *Arch Biochem* 11: 131, 1948.
- <sup>4</sup> NEUKIRCH P. Über die Jodophile Substanz der Leukocyten und ihr Verhalten zur Beutschen Färbung. *Ztschr f klin Med* 70: 251, 1910.
- <sup>5</sup> STAHL R. HORSTMANN AND HILSNITZ. Untersuchungen mittels der vitalen Jodfixation am strömenden Blute und am Knochenmark. *Vichows Arch f path Anat* 257: 392, 1915.
- <sup>6</sup> MANCINI R. E. AND CELANI BARRY R. Estudio critico del glucógeno en los leucocitos. *Rev Soc argent de biol* 17: 499, 1941.
- <sup>7</sup> — AND —. El glucógeno de los leucocitos. Fijado por el metodo de congelación y d. secación d. Altmann Gersch. *Rev Soc argent de biol* 18: 367, 1942.

- <sup>8</sup> MITCHEL, A J, AND WISLOCKI G B Selective staining of glycogen by ammoniacal silver nitrate Anat Rec. 90 261, 1944
- <sup>9</sup> WISLOCKI, G B, AND DEMPSEY E W Observations on the chemical cytology of normal blood and hemopoietic tissues Anat Rec 96 249, 1946
- <sup>10</sup> McMANUS J F A Oxidation of mucin with periodic acid producing the capacity to stain with Schiff's reagent Nature 158 202 1946
- <sup>11</sup> LILLIE, R D Reticulum staining with Schiff reagent after oxidation by acidified sodium periodate J Lab & Clin Med 32 910, 1947
- <sup>12</sup> ——— Studies on the preservation and histologic demonstration of glycogen Bull Internat A Med Museums No 27 p 23 1947
- <sup>13</sup> BAUER H Mikroskopisch-Chemischer Nachweis von Glykogen und einigen anderen Polysacchariden Ztschr f mikro anat Forsch 33 143 1933
- <sup>14</sup> GOOD C A KRAMER H AND SOMOGYI M The determination of glycogen J Biol Chem 100 484 1933
- <sup>15</sup> NELSON, N A photometric adaptation of the Somogyi method for the determination of glucose J Biol Chem 153 375 1944
- <sup>16</sup> SNAPPER, I MIRSKY A E RIS H, SCHNEID B AND ROSENTHAL, M Development of inclusion bodies containing ribose nucleic acid in myeloma cells after injection of stilbamidine Blood 2 311, 1942.
- <sup>17</sup> MEYER, K H The chemistry of glycogen Advances in Enzymology 3 109 1943
- <sup>18</sup> ELLIS R W B AND PAYNE W W Glycogen disease Quart J Med 5 31, 1936
- <sup>19</sup> BRIDGE E M AND HOLT L E Glycogen storage disease J Pediat 27 299 1945
- <sup>20</sup> VAN CREVELD S Investigations on glycogen disease Arch Dis Child 9 9 1934
- <sup>21</sup> WAGNER R The estimation of glycogen in whole blood and white blood cells Arch Biochem 2. 249 1946
- <sup>22</sup> WRIGHT J H The origin and nature of blood platelets Boston M & S J 154 643 1905
- <sup>23</sup> WAGNER R Studies on the physiology of the white blood cell Blood 2 235 1947

# A HISTOCHEMICAL STUDY OF ACID AND ALKALINE PHOSPHATASE DISTRIBUTION IN NORMAL HUMAN BONE MARROW SMEARS

By M. RABINOVITCH AND D. ANDREUCCI, M.D.

**A**LKALINE phosphatase activity was studied histochemically in normal and abnormal human blood and bone marrow smears (Wachstein<sup>1</sup>) after covering them with an alcohol-ether-celloidin solution for a few seconds. In a preliminary note we<sup>2</sup> reported the distribution of acid phosphatase in bone marrow smears after fixation in chilled acetone. Rheingold and Wislocki<sup>3</sup> reported on the localization of both phosphatases in smears and imprints of bone marrow after fixation in chilled 80 per cent alcohol. Preliminary work to determine the best fixation procedure led us to the conclusion that formol-vapor gives the best cytological preservation for both phosphatase techniques. In this paper we describe the distribution pattern of both phosphatases in normal human bone marrow smears using this method of fixation.

## MATERIALS AND METHODS

Bone marrow smears were obtained by sternal puncture from 17 normal pregnant women and 3 adult men without hematologic disorders. The smears were made on slides, rapidly dried in air and stored in a sulphuric acid desiccator until the time for fixation. In most instances they were subjected to the phosphatase methods 2-3 days after this puncture.<sup>4</sup> 15-30 day old slides yielded the same results but after two months storage a distinct reduction in activity was observed especially of the acid phosphatase activity. The methods used were those reported by Gomori<sup>5</sup> but somewhat modified by Wachstein<sup>1</sup> and ourselves.<sup>2</sup>

For the alkaline phosphatase technic the following incubation mixture was used: water, 10 ml, 6 per cent barbital buffer (pH 9.0-9.5), 5 ml, 2 per cent sodium glycerophosphate (50 per cent alfa May and Baker), 2 ml, 2 per cent calcium nitrate, 2 ml, and 1 ml of a 0.1 per cent magnesium sulphate solution. Slides were incubated from 10 to 20 hours at 37°C, then treated according to Wachstein<sup>1</sup> with due consideration for the critical timing studied by Danielli.<sup>6</sup> Controls were run by omitting the glycerophosphate solution from the incubation mixture.

For the acid technic the incubation mixture, treatment and controls were the same as those referred to in our previous note.<sup>2</sup> Incubation periods were from 10 to 20 hours or more. Incubation at pH 5.2 gave the same results as at pH 4.2. Dilution of the incubation mixture (2 or 1:4) did not interfere with the results.

**Choice of fixatives.** The following fixatives produced an intense or total inactivation of both phosphatases after incubation for 10 hours: Osmic acid vapor for 2 minutes then wash for 30 seconds; Bouin's solution for 30 minutes then wash 15 minutes; methyl alcohol for 4 minutes then wash 30 seconds. The following fixation methods preserved activity of both phosphatases: acetone at 4°C for 30 seconds then wash a few seconds; treatment according to Wachstein<sup>1</sup>; 10 per cent formol saline for 15 minutes then wash for 15 minutes; formol vapor for 3 minutes at 44°C, then wash in running water for 15 minutes; †, ‡, §.

From the Department of Obstetrics and Department of Histology and Embryology, Faculdade de Medicina da Universidade de São Paulo, São Paulo, Brazil.

\* Smears fixed 15 minutes, 5, 12 or 14 hours after the puncture yielded the same results.

† Washing for 100 minutes under the tap did not alter the results.

‡ Fixation for 10 minutes resulted in distinct inhibition of enzymatic activity especially of the alkaline enzyme. In the later stages of this investigation, 5 min. fixation was used for the acid technic; the greater inactivation so produced was counteracted by extension of the incubation period.

§ Fixation in a closed vertical Coplin jar containing a few drops of neutralized formaldehyde.

In addition to these 1 per cent formol in 50 per cent alcohol for 5 minutes then wash for 15 minutes and alcohol sublimate (1 part absolute alcohol and 1 part saturated aqueous sublimate solution) for 30 minutes then wash for 15 minutes inactivated the acid but partly preserved the alkaline phosphatase reaction

Ten per cent formol saline best preserved the enzyme activity of both phosphatases. The most satisfactory cytologic preservation in both methods was obtained by means of the formol vapor fixation and it was therefore the fixation procedure used throughout this study.

The photomicrographs were obtained from the richest and best preserved smears from cells whose nuclear pattern most nearly approached that obtained by means of the best fixation procedures with the usual hematologic stains. They do not therefore represent the commonest pattern observed.

The terminology used is that of Ferrata,<sup>7</sup> slightly modified with regard to the red cell series, in which we considered early and late erythroblasts.

## OBSERVATIONS

### *Acid Phosphatase*

About 240 slides were studied and of these 110 had been fixed by means of formol vapor. On the latter, 70 were considered good ones. Slides from each case were incubated on at least two different occasions.

*General data.* An evident correlation between the intensity of the reaction and cellular richness of the marrow material was found, but slight qualitative differences between slides were also observed. In some of the slides a dark extracellular precipitate occurred, which did not disturb the examination of the smears. The background in smears fixed by formol saline or formol vapor remained unstained, but after acetone fixation or Wachstein's treatment, it assumed a brownish color (extracted enzyme?). With incubation periods of about 10 hours the structures positive for acid phosphatase were distinctly stained and extension of the incubation periods (up to 33 hours, for instance) brought only a deeper stain, the qualitative picture remaining the same (see fig. 12).

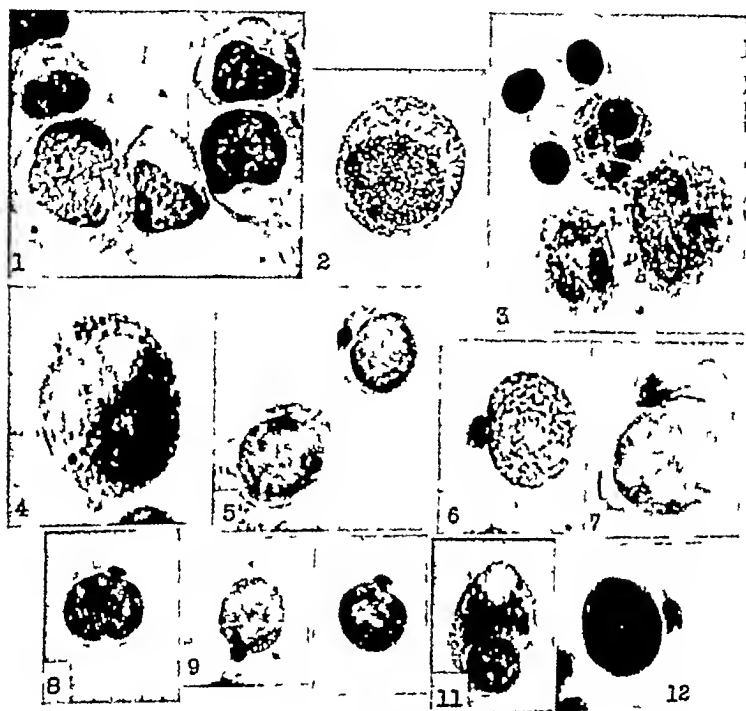
Nuclear pattern generally approached that obtained with hematologic stains and Feulgen's method, and in the best preserved smears conformed almost exactly to that pattern. When this occurred, a negative ground cytoplasmic reaction was observed in the granulocytic and in part of the red cell series. These were considered the most reliable acid phosphatase reaction pictures. We therefore will not describe the nuclear pattern of the best preserved smears. The intensity of the nuclear stain of the granulocytic and red cell series increased as maturation occurred parallel to what is observed with the Feulgen reaction.

Nonspecific granules were negative, appearing on the nuclei of immature cells (figs. 1, 2, 3). \* Specific neutrophilic (figs. 2, 3) and eosinophilic granules (fig. 4) reacted, although the former presented a variation in number and intensity of

\* The nonspecific granules referred to here are light areas in the basophilic cytoplasm over the nuclear area and beyond the nuclear membrane as seen in dry smears. They represent the negative images of cytoplasmic organoids—chiefly mitochondria. Depending upon their location they contribute to what hematologists have called hyaloplasm and parachromatin (See Jones O. P. Blood 3: 967, 1948). In a personal communication from M. Rabinovitch he more or less agrees with this interpretation. It seems that the term nonspecific granule should not be used for the negative images of organoids because the various aurophilic granules have been considered as nonspecific in contrast to the specific granules.

*Ed. O. P. J.*

staining This variation was observed only in material fixed by formol vapor, and in this particular case we could not blame fixation time, incubation period, or the intensity of the reaction

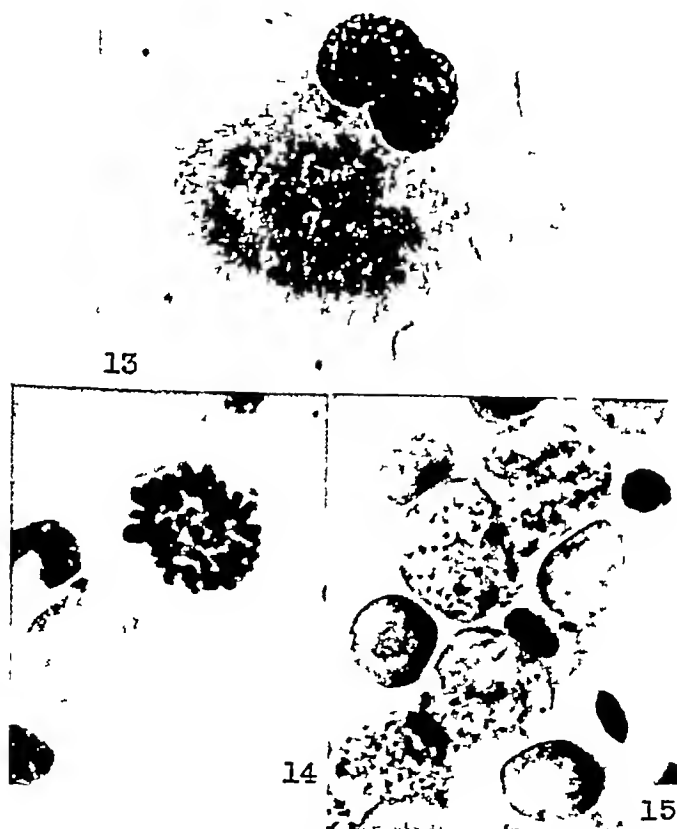


FIGS. 1-12.—PHOTOMICROGRAPHS OF HUMAN MARROW SMEARS AFTER ACID PHOSPHATASE REACTION Magnification 1940 diameters excepting figures 1 and 2 which are 1200 diameters Reduction about  $\frac{1}{3}$  off Fig 1—Promyelocytes with negative images of organoids over nucleus Reaction of neutrophilic granules is almost absent Fig 2—Neutrophilic promyelocyte with positive reaction of specific granules and negative images of organoids Fig 3—Neutrophilic promyelocyte and two segmented forms with positive specific granules and negative images of organoids Three erythroblasts with strongly positive nuclei Fig 4—Eosinophilic promyelocyte with granules in focus to show strongly positive reaction Figs 5, 6, and 7—Early erythroblasts showing a stronger reaction of the nucleus than cytoplasm with the exception of a well defined cytoplasmic reaction zone Figs 8, 9 and 10—Lymphocytes showing a strong reaction in the region of the Golgi element Fig 11—Plasmacyte with characteristic reaction Fig 12—Unidentified cell with strongly positive cytoplasmic reaction zone

Nucleoli (fig 2) did not stain or at least they reacted less intensively than the remainder of the nucleus They were frequently surrounded by a chromatin condensation which was very reminiscent of the nucleolus associated chromatin studied by Swedish authors

In other slides nuclear pattern was somewhat obscured and more uniform than that referred to above, although it did not prevent cell identification This was

especially true for mature neutrophils and erythroblasts, where the block nuclear pattern became indistinct. In a few slides, nuclear pattern was thread-like similar to that observed after acetone fixation. In these slides a slight to moderate cytoplasmic reaction of neutrophils and erythroblasts was observed, but other

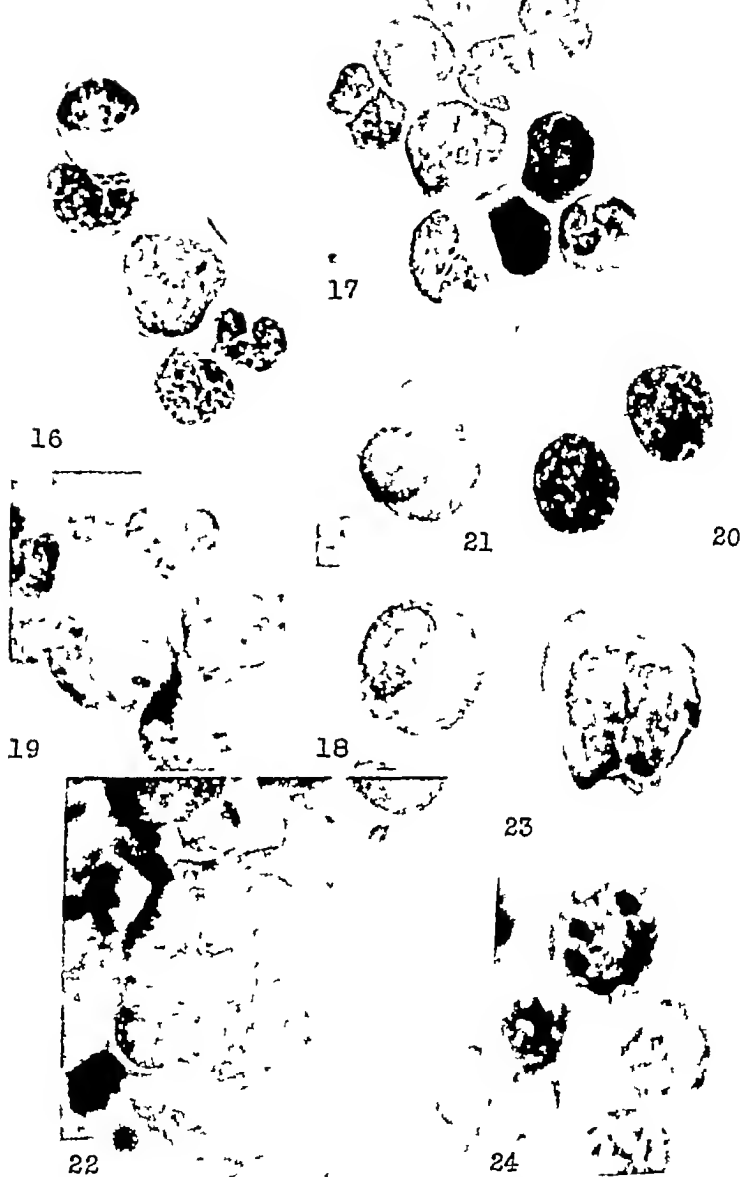


Figs 13-15—Photomicrographs of smears after acid phosphatase reaction. Magnification 1940 diameters except Fig. 13 which is 1200 diameters. Fig. 13—Megakaryocyte from human marrow showing a strongly positive reaction of both nucleus and cytoplasm. Fig. 14—Shows strongly positive reaction in mitotic figure and little or no reaction in cytoplasm. Fig. 15—Chick marrow smear showing the localization of a strongly positive reaction on granules within eosinophilic ones. Note the negative reaction of the adult red cell.

general points were coincident with the best preserved smears. Mitotic chromosomes (fig. 14) reacted strongly, in contrast with a negative or slightly reacting cytoplasm.

Part of the erythroblasts, lymphocytes, plasmacytes and megakaryocytes presented a localized cytoplasmic reaction that will be described below.





FIGS 16-24—PHOTOMICROGRAPHS OF HUMAN MARROW SMEARS AFTER ALKALINE PHOSPHATASE REACTION Magnification 1940 diameters excepting figs 16 and 17 which are 1600 diameters Reduction about  $\frac{1}{3}$  off Fig 16—Promyelocyte myelocyte and three segmented neutrophils two of which show a moderately positive cytoplasmic reaction Fig 17—Promyelocyte, metamyelocyte two stab forms and five segmented neutrophils Note cracks in some cells Fig 18—Eosinophilic metamyelocyte with negative reaction of specific granules Fig 19—Immature cells of neutrophilic series strongly positive nuclear reaction Fig 20—Early erythroblasts with nearly negative cytoplasmic reaction Fig 21—Plasmyocyte with strong nuclear and moderately diffuse cytoplasmic reaction Fig 22—Megakaryocyte with strong nuclear and nucleolar reaction and moderately diffuse cytoplasmic one Fig 23—Metaphase plate with poorly preserved chromosomes The extraction appearance of the cytoplasmic rim is

*Granulocytic series* Apart from variations in the staining intensity and number of neutrophilic granules referred to above, the general points were constantly encountered in well preserved smears. Cells of the eosinophilic series gave a conspicuous and constant reaction on specific granules—so much so that nuclear detail was sometimes obscured. It was difficult to recognize basophil leukocytes in our normal material. Cells of the granulocytic series are illustrated in figures 1-4.

*Red cell series* The general nuclear and cytoplasmic reactions were like those mentioned previously. In early erythroblasts localized zones of cytoplasmic reaction were frequently apparent (figs. 5-7). There was generally one of these zones but sometimes as many as three were encountered. These zones, which were frequently juxtanuclear in position and well delimited, were either irregular in shape or presented a number of closely arranged granules or rods, but their reaction was occasionally homogeneous. Mature red blood cells did not give a positive reaction.

*Lymphocytes* The nuclear pattern displayed by the reaction in lymphocytes was similar to the characteristic picture obtained with the usual hematologic stains. There were frequently one or two, and sometimes up to five cytoplasmic zones of reaction. Two general results were noted, in one type, the reacting zone was similar to that of some erythroblasts by being homogeneous and poorly defined, in the other and most frequent type the reaction was granular in nature. Sometimes granules were located on a small zone of reacting ground cytoplasm. The zones were frequently juxtanuclear in position, encircling the nucleus when 3 or more in number (figs. 8-10).

*Plasmacytes* Nuclear pattern of plasmacytes was frequently more homogeneous than after common hematologic stains. The cytoplasm was frequently vacuolated and of indistinct contour, in most instances, on a moderate ground cytoplasmic reaction, there were up to 10 small distinct, strongly reacting granules (fig. 11). In rare instances an entirely negative cytoplasm was found.

*Megakaryocytes* They presented a strong nuclear reaction with the usual pattern. In the cytoplasm of all elements there was a large, finely granulated strongly reacting zone with indistinct boundaries. This cytoplasmic zone was frequently juxtanuclear in position and it occasionally covered the nucleus. The remaining cytoplasm stained very lightly and its limits were not well marked (fig. 13). We did not observe well preserved platelets in the normal marrow material, therefore no reference is made to them.

Some cells that could not be identified with certainty, presented zones of cytoplasmic reaction similar to those of erythroblasts or lymphocytes (fig. 12).

#### *Alkaline Phosphatase*

One hundred and eighty smears were stained, about 50 being fixed in formol vapor. Results obtained were less satisfactory than those of the acid technic. Only about 40 per cent of the stained slides were considered good, with appreciable reaction on elements poor in the enzyme. A correlation was evident in the same material between the intensity of staining and incubation period, but comparably rich marrows presented unexplained differences in reaction intensity. As a rule cytologic preservation was not as good as that obtained by the acid tech-

analogous to that occasionally observed in Leishman stained smears. Fig. 14—Immature cell showing strongly positive nucleolar reaction after Wachstein's method. Note weakly positive nuclear reaction of adjacent forms.

Occasionally, cell fragmentation was encountered. After Wachstein's treatment, nuclear reaction was frequently negative, especially in erythroblasts and neutrophils (fig. 24), but the reaction was predominantly nuclear after formol fixation. Nuclear pattern was more homogeneous and blurred than that obtained with common stains and Feulgen reaction. Frequently especially in the neutrophilic series and erythroblasts, a finely granular pattern over a diffuse and homogeneous nuclear reaction was present. Both in granulocytic and red cell series the intensity of nuclear reaction was somewhat parallel to cell maturation. Cytoplasmic reaction was generally negative, except in part of the neutrophils and stab forms, and in plasmacytes. Nonspecific and specific neutrophilic and eosinophilic granules did not stain. Nucleoli reacted almost constantly (fig. 19). They were quite evident in immature cells but because the intensity of their reaction in promyelocytes was not very different from that of the remainder of the nucleus they were rarely demonstrated in these forms. After Wachstein's treatment and formol saline fixation, nucleolar reaction was very conspicuous and constant against a less intense nuclear stain (fig. 24) particularly in the eosinophilic and red cell series. Mitotic figures were also positive (fig. 23), although chromosome preservation was inferior to that obtained by the acid technic.

*Granulocytic series.* In agreement with Wachstein's report,<sup>1</sup> strongly reacting mature neutrophils and stabs were found in our normal material (figs. 16 and 17). In the majority of instances the nucleus and cytoplasm alike stained strongly so that nuclear details were entirely obscured, in other cells the reaction was only nuclear. No estimation of the number of strongly reacting neutrophils was made. This intense reaction on part of the neutrophils was evident in all slides, even in the poorest or least stained, where the remaining cell elements stained slightly. In the eosinophilic series, reaction was nuclear in location, no staining of the granules was ever observed (fig. 18).

*Red cell series.* Erythroblasts stained strongly in the nuclei, and in the best preserved slides they presented a nearly negative cytoplasm (fig. 20). In other slides, where nuclear pattern was less precise and more homogeneous, a slight to moderate cytoplasmic staining could be observed. Mature red blood cells did not stain.

*Plasmacyte series and lymphocytes.* Plasmacytes presented an homogeneous nuclear reaction (fig. 21) and in addition had a strongly reacting and homogeneous cytoplasm. Lymphocytes presented only nuclear reaction, their identification being generally difficult in view of the uniformity of the nuclear pattern.

*Megakaryocytes.* A strong and rather homogeneous reaction was given by the megakaryocytic nuclei. Frequently 10 or more nucleoli were clearly recognized, especially after Wachstein's treatment and formol saline fixation. The cytoplasm gave a moderate reaction (fig. 22). As platelets were not observed in good preservation, we refrain from commenting on them.

## DISCUSSION

### The Methods

Present status of the alkaline method was well discussed by Lison.<sup>8</sup> This author has confirmed and extended previous observations, supporting the specificity of

the method. Two points among others are significant (1) A negative reaction does not indicate the absence of the enzyme, (2) a slight diffusion of the enzyme can occur, thus hampering the interpretation of images on the cytologic scale. We think that under the conditions in which this work was performed the second possibility has been reduced to a minimum.

The specificity of the acid technic has been recently strongly criticized, at least for nervous tissues (Heinzen,<sup>9</sup> Lassek,<sup>10</sup> Bartelmez and Bensley<sup>11</sup>). Lison<sup>8</sup> emphasizes the necessity of a revision of the conditions of the reaction.

Results from this laboratory<sup>12</sup> tend to prove the specificity of the acid technic, for constant agreement has been obtained between chemical and histochemical data (frozen liver sections), in regard to the action of fixatives, the histologic procedure, the effect of temperature, and the inactivation by known enzyme inhibitors (NaF,  $\text{NH}_4\text{OH}$ ).

From the present work we concluded that formol vapor fixation is the procedure that gives the best cytological preservation for both phosphatase technics without extreme enzyme inactivation. In a cytologic study such as this, we think this is a very important point. For a discussion on the best histochemical fixation procedure we refer to Lison.<sup>13</sup>

As can be seen in the observations, the acid method gave more satisfactory and constant results than the alkaline. In the application of both technics we have confirmed the fact already established by Wachstein<sup>1</sup> for the "alkaline method, namely, a correlation between intensity of staining and cellular richness. The reason for this is unknown to us, but especially for the acid technic, the faint reaction of cells (particularly if poor in the enzyme) in poor materials could be partly overcome by extending the incubation period. It seems interesting to note that Fell and Danielli<sup>14</sup> in their study of alkaline phosphatase distribution in experimental wounds pointed to a somewhat similar relationship: mitotic chromosomes gave a stronger reaction where the intensity of staining of the sections was high and the inverse was true where the rate was low. Although in this case we cannot exclude the possibility of diffusion, as pointed out by Lison,<sup>8</sup> we do not think that this occurs in our case, for we generally studied isolated cells and the intercellular background was not stained.

#### *General Histochemical Data*

We shall adhere to general results on blood and bone marrow cell reaction, the staining of specific granules being considered under a separate heading. No thorough attempt will be made to review all papers in which incidental reaction of leukocytes was noted.

*Acid phosphatase.* Gomori<sup>5</sup> working with smears and sections evinced the negativity of all blood cells of all species studied, although round cell infiltrates around strongly positive areas showed some staining (diffusion?). Although Wislocki and Dempsey<sup>15</sup> stated (p. 254) they have studied bone marrow sections incubated at pH 5.0, they do not specify the results so obtained. In a previous note<sup>2</sup> we described the distribution of acid phosphatase in acetone fixed bone marrow smears. Rheingold and Wislocki<sup>3</sup> summarily reported results

obtained on monkey marrow smears and imprints after 80 per cent alcohol fixation. They noted the predominantly nuclear reaction of all cell series and a brownish granulation in the cytoplasm of myelocytes, in addition to diffuse nuclear and cytoplasmic staining. A variable staining for acid phosphatase of tissue lymphocytes has been noted by various authors (for instance Wolf et al.<sup>16</sup>, Wislocki and Dempsey<sup>15</sup>).

We have confirmed and extended the results summarized in our previous note,<sup>2</sup> but in view of the improvement of the results brought about in the fixation procedure and the extension of the materials, some points are to be modified, namely the positivity of nonspecific granules (see previous footnote) was not confirmed, neutrophilic granules stained, although variably, nuclear pattern, contrary to what we stated for acetone fixed smears, in the present material approximated that obtained by the Feulgen reaction. The progressive tendency to assume this pattern, verified by the use of progressively better fixation procedures led us, perhaps somewhat arbitrarily, to assume that this pattern probably conditions the most reliable figures of enzyme distribution, that was the starting point for our description.

*Alkaline phosphatase* Gomori<sup>4</sup> referred to the positivity of circulating blood granulocytes both in smears and sections. In 1943 the same author<sup>17</sup> noted a negative reaction of human leukocytes as observed in normal lung sections. Bourne<sup>18</sup> stated that nuclei of all marrow cells gave a positive reaction in sections, and that there seemed to be a greater staining in the primitive granulocytes than in the primitive red cells. Fell and Danielli<sup>14</sup> described intense cytoplasmic and nuclear reaction of neutrophils and nuclear reaction in migrating monocytes and lymphocytes in experimental wounds of the rat. Wislocki and Dempsey,<sup>15</sup> using monkey bone marrow sections stated that the reaction was spotty, seemingly involving cells in the neighborhood of blood vessels rather indiscriminately, instead of being visibly localized in any specific cell type.

Wachstein,<sup>1</sup> working on blood and bone marrow smears after treatment by an alcohol-ether-celloidin solution noted nuclear and/or cytoplasmic reaction of part of the neutrophils, the remaining cells being negative. Applied to bone marrow smears the technic was not as satisfactory as to blood smears. Not infrequently uneven staining was observed. Groups of cells showed activity while a similar type of cells, when more isolated in other fields, appeared to be devoid of phosphatase. Occasional nuclear staining of red cell series and late neutrophilic forms were noted. Megakaryocytes were, as a rule, negative.

Rheingold and Wislocki<sup>3</sup> studied alkaline phosphatase distribution in sections and imprints of the rhesus monkey bone marrow, it was present in the granulocytic series, especially in myelocytes, diffusely and variably present in cytoplasm and nuclei. Nucleated red cells did not stain. Cytoplasm of megakaryocytes showed a faint diffuse staining.

We agreed with Wachstein<sup>1</sup> on some correlation between cellular richness and staining intensity, although with frequent exceptions as different cases were compared and on the intense staining of part of the stabs and segmented neutrophils. But in many well preserved films all cellular marrow elements stained, therefore,

with the formol vapor fixation we could extend Wachstein's<sup>1</sup> observations and detail them in the cytologic plane.

We agreed with Rheingold and Wislocki<sup>2</sup> on the positivity of granulocytic and megakaryocytic series but could not confirm the negativity of the red cell series. Probably differences in the fixation procedures used can explain this disagreement.

The apparent discrepancies between results obtained by both phosphatase techniques in smears and sections are probably due to the great inactivation of both enzymes, particularly the acid one by fixation and by histologic procedures.<sup>3, 12, 19, 20</sup>

### *The Reaction of Cell Granules*

The constitution of specific granules is reviewed by Neumann<sup>21</sup> for the early literature. Jones<sup>22</sup> and Rheingold and Wislocki<sup>2</sup> reviewed part of the recent progress in this field. This work tends to prove that neutrophilic granules contain acid phosphatase—apart from the irregularity of the reaction—and do not give the alkaline reaction. The positivity of neutrophilic granules to the acid reaction was only apparent after formol fixations, the reaction being negative after Wachstein's treatment or acetone fixation. Unfixed slides also displayed a positive reaction on neutrophilic granules. Rheingold and Wislocki's<sup>2</sup> brownish granulation in myelocytes possibly represents reaction of specific granules. A positive reaction of neutrophilic granules was also found in frozen sections of human placenta treated by the acid technic (incubation period, 3 hours).

We confirmed our previous work<sup>2</sup> on the positive reaction of eosinophilic granules for acid phosphatase, such reaction was evident after Wachstein's treatment, formol and acetone fixations. These granules were negative for alkaline phosphatase. The positive reaction for acid phosphatase does not agree with previous work by Dempsey and Wislocki<sup>15</sup> and Wislocki et al.<sup>23</sup> on sectioned material. Rheingold and Wislocki<sup>2</sup> do not specify if the brownish granulation of myelocytes represents the reaction of eosinophilic granules. The negative reaction of these granules for alkaline phosphatase agrees with previous reports<sup>1, 3, 15, 23</sup> but does not agree with the report by Dalgaard and Dalgaard<sup>24</sup> on the positive reaction of granules of eosinophils in the intestinal mucosa of the rat. It is possible that differences in the techniques used may explain this discrepancy.

Unpublished results from this laboratory have also shown that rat bone marrow eosinophils, guinea pig eosinophils and pseudoeosinophils and chick eosinophils (with regard to their staining reaction) give the acid reaction on their granules. In chick granules (both round and fusiform) the reaction is confined to a central or eccentric well defined point (fig. 15), this localization closely resembles that of fuchsinophilic granules described within avian eosinophilic ones by Oriá<sup>25</sup> in this laboratory.

The positivity of tissue basophils for alkaline phosphatase was reported by previous authors,<sup>15, 24, 26, 27, 29b</sup> as well as that for the acid technic.<sup>2, 19, 23</sup> Since we could not identify basophils in our normal marrow material, we could not extend this to bone marrow elements.

### *The Positive Reaction of Mitotic Figures*

The positive alkaline phosphatase reaction (mitotic chromosomes) in bone marrow cells agrees with previous work on various materials <sup>14 18 20 21 22</sup>

Acid reaction of mitotic chromosomes has also been already described <sup>20 21</sup> All previous authors worked on sections of tissues fixed in acetone or 80 per cent alcohol

### *Reaction of Nucleoli*

This work confirms that of others on sections of various tissues, as to the positivity of nucleoli for the alkaline phosphatase technic <sup>14 20 21 22 23</sup> The negative reaction of nucleoli for the acid technic does not agree with the findings of Wachstein,<sup>20</sup> Bartelmez and Bensley,<sup>21</sup> Sullivan and Gardner<sup>21</sup> on nervous and hepatic tissues, and others. It is possible that these results were conditioned by the fixation procedure. It is of interest that in bone marrow smears fixed in formal saline occasionally there is a positive nucleolar reaction for acid phosphatase. The point is being investigated further.

### *The Negative Red Blood Cell Reaction for Acid Phosphatase*

This negative red blood cell reaction is striking in view of the known richness of red blood cells in this enzyme <sup>24</sup> It was observed after the various fixation procedures had been used. It may be that the enzyme is in the lyoenzyme form, and is extracted in the fixation procedure, or that the negative reaction is due to the nonpenetration of the ions used in the method. In chick's mature red blood cell acid phosphatase was localized exclusively on the nucleus (fig. 15). This agrees with chemical data of Dounce and Seibel <sup>25</sup>

### *Acid Phosphatase Reaction of Cytoplasmic Zones of Lymphocytes, Plasmacytes, and Erythroblasts*

*Lymphocytes* The reaction, although not constant, strikingly suggests the appearance of the Golgi zone of lymphocytes as described and pictured by Richter <sup>26</sup> Compare, for instance, his figures 31, 33, 34, and 35 with our figures 8, 9, and 10. This would be another example of reaction of the Golgi zone for alkaline and acid phosphatase that has been described<sup>22 27 28 29</sup> in a variety of tissues. In the last paper, Deane<sup>29</sup> stated that the same fact was found in tissue polymorphonuclear leukocytes, but did not give any detail. These authors discussed the possible functional significance of the phosphatase reaction of the Golgi zone of various tissues. Paff et al.,<sup>30</sup> in a cytochemical study of normal and malignant mast cells, described and illustrated positive zones of cytoplasmic acid phosphatase reaction and interpreted them as the Golgi apparatus. In lymphocytes, it is tempting to relate it to the probable globulin synthesis by these cells (see, for instance, White and Dougherty<sup>40</sup>). A study of lymphocytes in malignant and virus processes should be of interest.

*Plasmacytes* The cytoplasmic reaction of plasmacytes, on the other hand, does not fit the description of the Golgi apparatus of plasmacytes by Estable<sup>41</sup> and by Ito,<sup>42</sup> both working on sections. The results we report, therefore, await further

interpretation. It is of interest that plasmacyte cytoplasm gives a strong reaction for both phosphatases as these cells have a cytochemical organization pointing to intense nucleoprotein metabolism and protein synthesis.<sup>43</sup>

*Erythroblasts* There is a striking similarity between some of our figures (for instance, fig. 6) and those of Jones<sup>44</sup> of the so called hyaloplasm of primitive erythroblasts after various staining procedures (see, for instance, his figs. 6, 9 and 12, plate 1). Hyaloplasm was shown by this author to represent negative images of mitochondria and probably of the Golgi element. Our figures do not coincide entirely with Cowdry's<sup>45</sup> (his figs. 10 and 20, for instance) of the Golgi apparatus of erythroblasts of guinea pig marrow. Unpublished work with F. T. Mendes has shown that promegaloblasts and part of the basophilic megaloblasts of megaloblastic anemia marrow present conspicuously reacting cytoplasmic zones similar to those of early erythroblasts.

### *Concluding Remarks*

The essentially qualitative results obtained by means of the phosphatase techniques do not permit us to relate our results with any certainty to the qualitative ones on thymonucleic acid,<sup>46</sup> ribonucleic acid (see White<sup>47</sup>), and to the quantitative data on nucleoproteins and proteins of Thorell.<sup>48</sup> The similarity of the acid phosphatase—and to a certain extent also of the alkaline reaction—nuclear pattern to that obtained after Feulgen's stain is suggestive in this connection.

Relationship of phosphatases to nucleoprotein metabolism was suggested by Bourne,<sup>18</sup> Bodian and Mellors,<sup>49</sup> Dempsey and Wislocki,<sup>50</sup> Moog<sup>51</sup> and others. More recent work by Brachet and Jenner,<sup>52</sup> on the parallel between intensity of alkaline phosphatase staining of nuclei of various tissues and turnover of thymonucleic acid phosphorus, and by Montalenti and De Nicola,<sup>53</sup> on the correspondence between alkaline phosphatase nuclear distribution and Feulgen's reaction of gonads of crustaceans, strongly points to that relationship.

The possibility that phosphatases participate in other metabolic lines has also been pointed out by some of the first mentioned authors. Chemical determination of at least the alkaline phosphatase activity if coupled with cellular content estimation of bone marrow should be of value and could be related to chemical data on nucleoproteins such as that of Davidson et al.<sup>54</sup>

### SUMMARY

1. Three minute fixation in formol vapor at 44°C, followed by 15 minute washing proved to be the most satisfactory fixation procedure for both acid and alkaline phosphatase techniques as applied to bone marrow smears.
2. For both techniques a relation between staining intensity and cellular richness was found.
3. The reaction of normal human bone marrow cells to both phosphatase techniques is described. Both are predominantly nuclear in location. Nuclear pattern approached that observed with common staining methods and Feulgen's reaction. Cytoplasmic reaction was nearly negative. Nonspecific and specific granules do not stain after the alkaline technic. Nonspecific granules are negative for acid



phosphatase, while specific neutrophilic are variable, and eosinophilic, constantly positive Nucleoli are negative after the acid technic, being positive for the alkaline enzyme Mitotic chromosomes are positive for both technics Acid phosphatase reaction in cytoplasmic zones of lymphocytes, erythroblasts, plasma cells and megakaryocytes, is described

## ACKNOWLEDGMENTS

Many points pertaining to this paper were discussed with the late Dr José Oriá to whom we owe invaluable stimulation and guidance We are indebted to Prof W Bnno of Montevideo (Uruguay) for kind advice on the Golgi apparatus of blood cells We are deeply indebted to Professor O P Jones of Buffalo for his painstaking help and advice We thank Dr M A Jamra (Section of Hematology Central Laboratory Hospital das Clinicas da Faculdade de Medicina) and Dr J F Pontes (Section of Clinical Therapeutics Hospital das Clinicas da Faculdade de Medicina) for part of the material used in this study We thank Messrs L. Frankenthal and G Lorenzoni for their aid in the preparation of some of the microphotographs

## REFERENCES

- 1 WACHSTEIN M Alkaline phosphatase activity in normal and abnormal human blood and bone marrow smears *J Lab & Clin Med* 31 1, 1946
- ✓ 2 RABINOVITCH, M, JUNQUEIRA L C U, AND MENDES F T Cytochemical demonstration of acid phosphatase in bone marrow smears *Science* 107 322, 1948
- 3 REINGOLD, J J, AND WISLOCKI G B Histochemical methods applied to hematology *Blood* 3 641, 1948
- 4 GOMORI G The distribution of phosphatase in normal organs and tissues *J Cell & Comp Physiol* 17 71 1941
- 5 — Distribution of acid phosphatase in tissues under normal and under pathologic conditions *Arch Path* 32 189 1941
- 6 DANIELLI, J F A critical study of techniques for determining the cytological position of alkaline phosphatase *J Exper Biol* 22 110 1946
- 7 FERRATA, A *Le Emopatie* Soc Ed Libreria Milano Vol 1 1933
- 8 LISON L La recherche histochemique des phosphatases Étude critique *Bull d histol appliq a la physiol* 25 23 1948
- 9 HEINZEN, B Acid phosphatase activity in transected sciatic nerves *Anat. Rec.* 98 193 1947
- 10 LASEK, A M The stability of so-called axonal acid phosphatase as determined by experiments on its stainability *Stain Technol* 22 133 1947
- 11 BARTELMER G W., AND BENSLEY S H Acid phosphatase reactions in peripheral nerves *Science* 106 639 1947
- 12 RABINOVITCH, M, FAJER, A B MANISADJIAN A AND JUNQUEIRA L C U To be published
- 13 LISON L Histochemie animale Methodes et problemes Paris Gauthier Villars 1936
- 14 FELL, L H B AND DANIELLI J F The enzymes of healing wounds I The distribution of alkaline phosphomonoesterase in experimental wounds and burns in the rat *Brit J Exper Path* 24 196 1943
- 15 WISLOCKI G B AND DEMPSEY E W Observations on the chemical cytology of normal blood and hemopoietic tissues *Anat Rec* 96 249 1946
- 16 WOLF A, KABAT E A, AND NEWMANN W Histochemical studies on tissue enzymes III A study of the distribution of acid phosphatases with special reference to the nervous system *Am J Path* 19 423 1943
- 17 GOMORI, G Calcification and phosphatase *Am J Path* 19 197 1943
- 18 BOURNE, G The distribution of alkaline phosphatase in various tissues *Quart J Exper Physiol* 32 1 1943
- 19 MONTAGNA W AND NOBACK C R Acid phosphatase and lipids in the mast cells of the rat *Science* 106 19 1947

- <sup>20</sup> STAFFORD R. O., AND ATKINSON W. B. Effect of acetone and alcohol fixation and paraffin embedding on activity of acid and alkaline phosphatases in rat tissues. *Science* 107 279, 1948
- <sup>21</sup> NEUMANN A. Über den gegenwärtigen Stand Unserer Kenntnisse über die chemische Beschaffenheit der Leukozytengranula. *Folia haematol.* 36 95, 248 and 463, 1928
- <sup>22</sup> JONES, O. P. Morphologic hematology. *Blood*, supp. 1 3, 1947
- <sup>23</sup> WISLOCKI G. B., BUNTINO H., AND DEMPSEY, E. W. Further observations on the chemical cytology of megakaryocytes and other cells of hemopoietic tissues. *Anat. Rec.* 98 527, 1947
- <sup>24</sup> DALOGAARD, E., AND DALOGAARD J. B. Om Mastceller og Deres Indhold af Fosfatase. En histologisk og histokemisk Undersøgelse. *Ugeskr. f. læger* 18 1, 1948
- <sup>25</sup> ORIA J. Sobre os Granulocytos Eosinophilos do sangue circulante da Amazona aestiva. *Ann. Fac. de Med. da Univ. da São Paulo* 4 3, 1930
- <sup>26</sup> MONTAGNA W. AND NOBACK, C. R. The histochemistry of the preputial gland of the rat. *Anat. Rec.* 96 111, 1946
- <sup>27</sup> NOBACK C. R., AND MONTAGNA W. Some histochemical aspects of the mast cell with special reference to alkaline phosphatase and cytochrome oxidase. *Anat. Rec.* 96 279, 1946
- <sup>28</sup> KRUEHLIS E. J. Cytological demonstration of phosphatase in chromosomes of mouse testes. *J. Cell & Comp. Physiol.* 19 376, 1942
- <sup>29</sup> WILMER, E. N. The localization of phosphatase in cells in tissue cultures. *J. Exper. Biol.* 19 11, 1942
- <sup>30</sup> WACHSTEIN, M. Influence of dietary deficiencies and various poisons on the histochemical distribution of phosphatase in the liver. *Arch. Path.* 40 57, 1945
- <sup>31</sup> SOLKIN N. M., AND GARNER J. H. The acid and alkaline phosphatase activity in the normal and recovering liver of the rat. *Anat. Rec.* 100 143, 1948
- <sup>32</sup> MOORE F. Localization of alkaline and acid phosphatases in the early embryogenesis of the chick. *Biol. Bull.* 86 51, 1944
- <sup>33</sup> MONTALENTI G. AND DE NICOLA, M. Comportamento delle fosfatasi alcaline all'azione di alcuni veleni citotossici. *Experientia* 4 314, 1948
- <sup>34</sup> ROCHE, J. Blood—phosphatases. *Biochem. J.* 25 1724, 1931
- <sup>35</sup> DOUNCE, A. L., AND SEIDEL, D. Acid phosphatase content of nuclei of chicken erythrocytes. *Proc. Soc. Exper. Biol. & Med.* 54 22, 1943
- <sup>36</sup> RICITER, K. M. An experimental study of the cytology of human peripheral blood neutrophils and lymphocytes. *J. Morphol.* 71 53, 1942
- <sup>37</sup> EMMEL, V. Alkaline phosphatase in the Golgi zone of absorbing cells of the small intestine. *Anat. Rec.* 91 39, 1945
- <sup>38</sup> DEANE, H. W., AND DEMPSEY, E. W. The localization of phosphatases in the Golgi region of intestinal and other epithelial cells. *Anat. Rec.* 93 401, 1945
- <sup>39</sup> ———. A cytochemical survey of phosphatases in mammalian liver, pancreas and salivary glands. *Am. J. Anat.* 86 321, 1947
- <sup>40</sup> PAPP G. K., MONTAGNA W. AND BLOOM, S. Cytochemical studies of normal and malignant mast cells. *Cancer Research* 7 798, 1947
- <sup>41</sup> WHITE, A. AND DOUGHERTY, E. F. The role of lymphocytes in normal and immune globulin production, and the mode of release of globulin from lymphocytes. *Ann. N. Y. Acad. Sci.* 46 859, 1946
- <sup>42</sup> ESTABLE C. Observations sur l'appareil de Golgi dans les cellules du sang et des organes hématopoïétiques. *Compt. rend. Soc. de biol.* 106 841, 1931
- <sup>43</sup> ITO T., TAKAHASHI T. AND MIZITANI Y. Über den Golgiapparat der Plasmazelle, mit besonderer Berücksichtigung auf das Wesen des hellen Hofes derselben. *Folia anat. japon.* 16 303, 1938
- <sup>44</sup> BINO, J. F., FAUREAU A., AND THORELL B. Studies on nucleic acid metabolism in plasma cells. *Acta physiol. Scandinav.* 10 282, 1945
- <sup>45</sup> JONES O. P. Mitochondria and their relation to so-called hyaloplasm. *J. Lab. & Clin. Med.* 32 700, 1947
- <sup>46</sup> COWDREY E. W. The reticular material of developing blood cells. *J. Exper. Med.* 33 1, 1921
- <sup>47</sup> LA COUR L. F. Mitosis and cell differentiation in blood cells. *Proc. Roy. Soc. Edinburgh* 62 73, 1944
- <sup>48</sup> WHITE J. C. The cytoplasmic basophilia of bone marrow cells. *J. Path. & Bact.* 59 223, 1947

- <sup>48</sup> THORELL, B. *Studies on the formation of cellular substances during blood cell production* London, Henry Kimpton 1947
- <sup>49</sup> BOGIAN D. AND MELLORS R. C. The regenerative cycle of motoneurons with special reference to phosphatase activity *J Exper Med* 81 469 1945
- <sup>50</sup> DEMPSEY E. W. AND WISLOCKI G. B. Histochemical contributions to physiology *Physiol Rev* 26 1 1946
- <sup>51</sup> MOORE, F. The physiological significance of the phosphomonoesterases *Biol Rev* 21 41 1946
- <sup>52</sup> BRACHET, J. AND JENNER R. Phosphatase alcaline des noyaux et vitesse de remplacement du phosphate de l'acide thymonucleique *Compt rend Soc de biol* 140 1121 1946
- <sup>53</sup> MONTALENTI G. , AND DE NICOLA M. Distribuzione di fosfatase alcalina in gonadi di Crostacei isopodi in rapporto col ciclo degli acidi nucleici *Experientia* 4. 315, 1948
- <sup>54</sup> DAVIDSON J. B. , LESLIE, I. , AND WHITE J. C. The distribution of nucleic acids in bone marrow cells, the distribution of nucleic acids *J Path & Bact* 60 1 1948

# PRODUCTION OF CHARCOT-LEYDEN CRYSTALS FROM EOSINOPHILS WITH AEROSOL MA

By COMMANDER W W AYRES, MC, U S NAVY

CHARCOT-LEYDEN crystals were first described in 1853 by Charcot,<sup>1</sup> and again in 1872 by Leyden.<sup>2</sup> They still remain enigmatic structures, and there are many conflicting reports in the literature as to their nature and significance. Their chemical nature is undetermined and there is considerable question as to whether they may be formed from normal blood. Thus, Liebreich<sup>3</sup> stated they may be produced from every normal human blood. Also Neumann<sup>4</sup> was able to produce them in normal blood. On the other hand, Thompson and Paddock<sup>5</sup> in a study of the blood of 100 routine hospital admissions found no Charcot-Leyden crystals. There is also considerable question as to whether all eosinophils are capable of forming crystals and whether they are specific for eosinophils. Schwarz<sup>6</sup> stated that the crystals are not an essential component of the eosinophil, since they could not be produced from the blood of all patients with eosinophilia. Again there are reports of the presence of crystals in the absence of eosinophils. Up to the present time there has been no method by which the crystals could be produced with certainty from eosinophils.

It is known that the crystals usually occur in association with eosinophils and that they are remarkably resistant to certain deleterious influences. Harrison<sup>7</sup> has isolated the crystals in pure form from minced leukemic spleens containing the crystals. The crystals have been described in a diverse number of diseases. In the sputum of asthmatics, in leukemic blood and tissues, in allergic nasal polyps, in the blood and tissues of patients with periarthritis nodosa, in the feces in amebiasis, in the feces in helminthiasis, and in the bone marrow of sickle cell anemia. The crystals appear limited to primates, there is only one questionable case in which they were reported in the blood of a frog.

The purpose of this paper is to present a method by which Charcot-Leyden crystals may be produced rapidly and with certainty from eosinophils by means of Aerosol MA\*, and to show that the crystals may be produced in the blood of a high percentage of normal persons and routine hospital admissions.

## METHOD

Four and one half cc of blood obtained by venipuncture is mixed with 0.5 cc of 3.8 per cent solution of sodium citrate. The blood is centrifuged and the buffy coat is removed by means of a capillary pipet. Two separate drops of this buffy coat are placed on a microslide. One of the drops is covered with a cover slip containing Aerosol MA and the other drop with a plain cover slip. According to Beeler,<sup>8</sup> Aerosol MA is dihexyl sodium sulfosuccinate. It is a homologue of Aerosol AY, Aerosol IB, and Aerosol OT and is a commercially pure white waxlike compound which is somewhat hygroscopic. The solu-

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\*American Cyanamid Co.

bility is 34.3 grams per 100 cc. of water at 25 C. It has been used as a wetting agent, emulsifying agent and detergent. Aerosol MA is rubbed over about half of the surface of the cover slip. The waxlike nature of the compound permits it to adhere readily to the surface. The cover slips are rimmed with petrolatum and the preparation is placed in a moist chamber to prevent drying. It is observed every twenty-four hours for seven days and the presence or absence of Charcot-Leyden crystals recorded.

The red cells contained in the buffy coat lyse immediately on application of the cover slip containing Aerosol MA, as do most of the leukocytes. The granules of the eosinophils and the Charcot-Leyden crystals do not lyse. The cells in the control preparations show little or no lysis for several days, and then as a result of bacterial growth. In the Aerosol MA preparations Charcot-Leyden crystals form within a few minutes to several hours while in the control preparations an appreciable number of crystals do not form for seventy-two hours.

Permanent preparations may be made by removing the cover slip with a sliding motion, drying in air, fixing for one minute with absolute methyl alcohol, and staining with the usual hematoxylin-eosin technic. The eosin should be slightly acidified for brilliant coloration.

### RESULTS

The blood of 100 routine hospital admissions was studied by this method and the results are shown graphically in figure 1. In the experimental group, in which the buffy coat was exposed to Aerosol MA, Charcot-Leyden crystals formed in 99 per cent of the cases by the first day. These all remained positive on the second, third and fourth days. On the fifth day one slide became negative for crystals to give a value of 98 per cent. Another slide became negative on the sixth and seventh days to give a final value of these last 2 days of 97 per cent. In the control group, none of the slides were positive the first day, 3 were positive the second day, 12 on the third day, 44 on the fourth day, 64 on the fifth day, and 75 on the sixth and seventh days. The figure 75, however, does not give a true value for the total number of cases that were positive, since on the latter two days as some slides became positive, others became negative. Actually, 80 per cent of the control group showed Charcot-Leyden crystals at one or more times.

In no case in the experimental group were Charcot-Leyden crystals found in the absence of eosinophils. In the control group, most of the cases which failed to show Charcot-Leyden crystals did show eosinophils.

It should be emphasized that the experimental preparations contained crystals in large numbers, directly proportionate to the number of eosinophils, and that the control preparations never showed crystals in the numbers seen in the corresponding experimental preparations.

The 100 hospital admissions were unselected and had a variety of diseases, such as hypertension, diabetes mellitus, Hodgkin's disease, abscess of the prostate, varicose veins, pneumonia, and fractures. Thirty-seven per cent of the patients had a total white count of over 10,000. In no patient was the total white count under 5,000. In 12 per cent of the patients there was an eosinophilia of over 5 per cent, the highest being 12 per cent. In 14 per cent of the cases the diagnosis was not determined. In the remaining 86, only 5 had the diagnosis of a possible allergic disease. There was no case of asthma, allergic rhinitis, or serum sickness.

The blood of 24 normal students was studied with the same technic, the results of which are shown in figure 2. In the Aerosol MA preparation 90 per cent were positive the first day for Charcot-Leyden crystals and remained positive throughout

the seven days of observation. In the control preparations, none of the slides were positive on the first and second days, 87 per cent were positive on the third day,

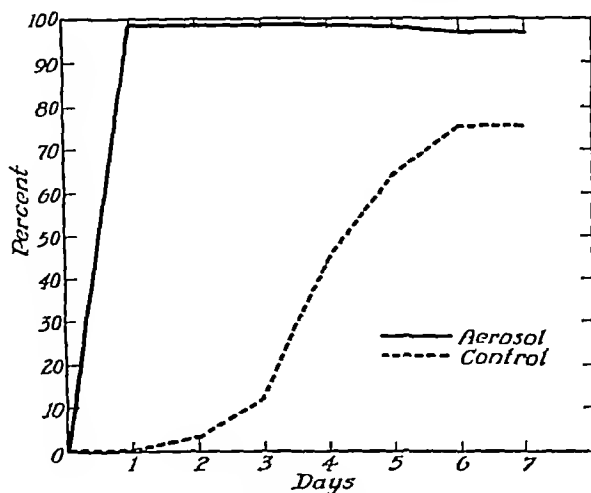


FIG 1.—CHARCOT LEYDEN CRYSTALS IN AEROSOL AND CONTROL PREPARATIONS IN 100 ROUTINE HOSPITAL ADMISSIONS

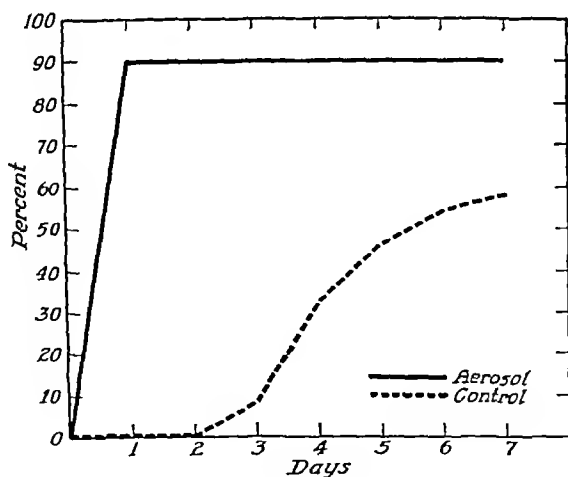


FIG 2.—CHARCOT LEYDEN CRYSTALS IN AEROSOL AND CONTROL PREPARATIONS IN 24 NORMAL PERSONS

31 per cent on the fourth day, 46 per cent on the fifth day, 54 per cent on the sixth day, and 58 per cent on the seventh day. All the control and experimental slides remained positive throughout the seven days.

The shape of the Charcot-Leyden crystal is that of a hexagonal pyramid with bases opposed, as shown in figures 3 and 4. Their hexagonal shape in cross section is well illustrated in figure 5. This is a paraffin section of eosinophilic granuloma of bone, stained with Gram-Weigert's stain. Their size is ordinarily described as from 7 to 21 microns in length. In this work some crystals were just visible with the oil immersion lens, while others measured 96 microns in length and 9 microns in width. The crystals stain black with iron-hematoxylin and red with hematoxylin-eosin. In fresh preparations they are colorless or have a light yellow tint.

Many observations were made to determine the origin of Charcot-Leyden crystals. They arise in two ways, intracellularly and extracellularly. Most of the crystals arise within the cell. The crystals elevate, then puncture the cell membrane. More than one crystal may be formed within a cell. It is the usual picture with the

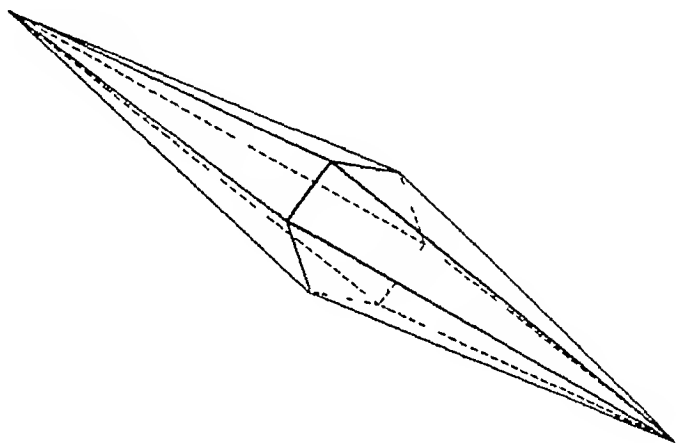


FIG. 3.—DIAGRAM OF CHARCOT-LEYDEN CRYSTAL

Aerosol MA method to see almost every eosinophil with one or more Charcot-Leyden crystals protruding from the cell. Often the crystals lie alongside the cell. Those crystals that arise extracellularly, first appear as minute crystals which gradually increase in size.

It is significant that, with the exception of the eosinophil granules and the Charcot-Leyden crystals, all formed elements of the blood are lysed, indicating that these granules and crystals are remarkably resistant to reduction in surface tension. The nucleus of the eosinophil is dissolved by Aerosol MA, since even after brief exposure to Aerosol MA, the nucleus of the eosinophil appears washed out and does not stain with Wright's stain. In control preparations, even after several days, some chromatin of the nucleus of the eosinophil takes the stain. There is no apparent reduction in eosinophil granules, nor change in their staining reaction, indicating that the crystals do not arise from the granules. It is probable then that the Charcot-Leyden crystals arise from the nucleus of the eosinophil, although this has not been proved.

It was stated by Schwarz<sup>8</sup> that the Charcot-Leyden crystal is not an essential

component of the eosinophil. Thus, Brown<sup>9</sup> studied the blood of a patient with trichiniasis who had 68 per cent eosinophils and was unable to produce Charcot-Leyden crystals. In our work, however, whenever eosinophils were found in blood



FIG 4—CHARCOT LEYDEN CRYSTAL IN WET PREPARATION X900



FIG 5—CROSS SECTION OF CHARCOT LEYDEN CRYSTAL SHOWING HEXAGONAL SHAPE X900

or in tissue, they could be made to produce Charcot-Leyden crystals with Aerosol MA. Conversely, no crystals were found in blood or tissue in which eosinophils were absent. Also the number of crystals formed was directly proportional to the



number of eosinophils present. Preliminary work indicates that the Charcot-Leyden crystal is not only an essential component of the eosinophil, but it is also specific for the eosinophil.

In patients with considerable eosinophilia, the crystals may be demonstrated in whole blood. The technic is the same as described, except a drop of blood obtained from a small wound of the finger is used instead of the buffy coat. In one patient with eosinophilia of 60 per cent, of unknown origin, associated with transient erythematous swellings of the subcutaneous tissues, crystals of large size could be demonstrated in the peripheral blood in a few minutes by use of Aerosol MA.

Another patient with embryonal carcinoma of the testicle metastatic to the lungs was studied. He had a pleural effusion containing about 95 per cent eosinophils.



FIG. 6—CHARCOT-LEYDEN CRYSTALS SHOWING BLUNT ENDS. X900

The centrifugate of this fluid was then almost pure eosinophils. When this centrifugate was mixed with Aerosol MA, very large abnormal crystals with blunt ends were formed, together with normal crystals. The largest of the crystals measured 96 microns in length and 9 microns in width (figure 6). It is believed that the malignancy was not responsible for the formation of the abnormal crystals. The more logical explanation is that the substance which forms Charcot-Leyden crystals was here present in such a large amount and subject to the influence of Aerosol MA that unusually large and abnormal crystals were formed.

A study was made of a patient with discoid lupus erythematosus of the face of thirty years' duration during an acute exacerbation. He showed an eosinophilia of 36 per cent. The buffy coat of the blood of this patient when mixed with Aerosol MA formed crystals within two minutes, the largest of which measured 200 microns in length. This represents about ten times the usual size of Charcot-Leyden crystals. Some of these crystals were remarkable in that they were fused in their

centers. The control preparation showed crystals in two days. On treatment with Bismarsen intramuscularly, the patient rapidly improved, the rash almost entirely disappeared, the eosinophils decreased to 6 per cent and lost their ability to form Charcot-Leyden crystals rapidly and of the abnormally large size. The inference is that some test based on the size and time of appearance of the crystals may be evolved by which the progress of allergic diseases can be determined.

### DISCUSSION

Reports in the older literature repeatedly emphasize that for the production of Charcot-Leyden crystals, the preparations must stand for a considerable period of time. It is also emphasized that large numbers may be found in the bone marrow of cadavers showing autolytic changes. In these cases there is almost always growth of bacteria and the release of ferments by the destruction of cells during autolysis.

It is believed then that the mechanism for the formation of Charcot-Leyden crystals relates to the destruction of the eosinophil by these various lytic agents. In this work, the control preparations did not become positive until the bacterial growth was quite heavy, and the majority of red cells and white cells showed degenerative changes. It is then believed that the mechanism of the action of Aerosol MA is related to its marked lysing effect due to its ability to lower surface tension. The conflicting reports in the literature then become understandable, in those cases in which the eosinophils were subject to lytic influences either by bacterial or enzymatic action, large numbers of crystals were produced, while the reverse happened if they were not subject to such influences. By the use of Aerosol MA these lytic factors may be controlled, with production of crystals from eosinophils in all tissues in which eosinophils were found.

Of particular interest is the work of Turner et al.<sup>10</sup> on the relationship of the eosinophil to the Gordon phenomenon. They showed that the agent producing encephalitis in rabbits was found only in the presence of the eosinophils. They also showed that the test was only positive in those cases of Hodgkin's disease in which eosinophils were found in the tissue. The exact nature of the exciting agent is unknown. Turner and his co-workers suggest that it is related to the Charcot-Leyden crystal. Support for this theory is that for the production of the agent, the tissue must autolyze in the refrigerator for 1 to 2 weeks to obtain a positive test—a factor favoring the production of Charcot-Leyden crystals.

At present this method of producing Charcot-Leyden crystals by means of Aerosol MA has no practical significance. It should simplify, however, the isolation of the crystals, the determination of their chemical structure, and thus lead to a better understanding of the eosinophil and the diseases with which it is associated.

### CONCLUSIONS

1. A method using Aerosol MA is presented by which Charcot-Leyden crystals may be formed from eosinophils with certainty, rapidity, and in quantity.

2. With the Aerosol MA method Charcot-Leyden crystals were demonstrated in 99 percent of the blood of 100 routine hospital admissions, the crystals were demonstrated in 80 per cent of the control group. With the Aerosol MA method

Charcot-Leyden crystals were demonstrated in 90 per cent of normal persons, the control group was positive for crystals in 58 per cent

3 Preliminary work indicates that the Charcot-Leyden crystal is not only an essential component of the eosinophil, but it is specific for the eosinophil

#### ACKNOWLEDGMENT

I wish to thank E. L. Elworth HMC USN, and W. O. Harrison HMC, USN for technical assistance in this work and M. N. Starkey for the photomicrography

#### REFERENCES

- <sup>1</sup> CHARCOT, J. M. AND ROBIN, C. Observation de leucocythemie. *Mem. de Soc. de Biol.* 5: 44-50 1853
- <sup>2</sup> LEYDEN, E. Zur Kenntnis des Bronchial Asthma. *Virchows Arch. f. path. Anat.* 54: 324-352, 1872.
- <sup>3</sup> LIEBREICH, E. In vitro Versuch über Eosinophilie. *Klin. Wchnschr.* 2: 194-198 1923
- <sup>4</sup> NEUMANN, A. Ueber die Natur der Charcot-Leyden Böttcher-Neumann Krystalle. *Ztschr. f. phys. Chem.* 173: 69-71 1917
- <sup>5</sup> THOMPSON, J. H. AND PAODOCK, F. H. The significance of Charcot-Leyden crystals. *New England J. Med.* 223: 936-939 1940
- <sup>6</sup> SCHWARZ, E. Die Lehre von der allgemeinen und örtlichen Eosinophilie. *Ergebn. d. allg. Path. u. path. Anat.* 27: 137-789 1914
- <sup>7</sup> HARRISON, G. A. *Chemical Methods in Clinical Medicine*. New York: Grune & Stratton 1947. P. 544
- <sup>8</sup> BEBLER, E. C. Hydrophilic Ointments and Bases. I. Compilation of newer ingredients with an index to trade names. *Bull. Nat. Formulary Committee* 28: 173-232, 1942.
- <sup>9</sup> BROWN, R. R. Experimental studies on the origin of Charcot-Leyden crystals with an account of their chemistry, occurrence and diagnostic importance. *Philadelphia M. J.* 2: 1076 1898
- <sup>10</sup> TURNER, C. J., JACKSON, H. AND PARKER, F. The etiologic relation of the eosinophil to the Gordon phenomenon in Hodgkin's disease. *Am. J. M. Sc.* 195: 27-32 1938

## EOSINOPHILIC LEUKEMIA

REPORT OF A CASE WITH AUTOPSY CONFIRMATION, REVIEW OF THE LITERATURE

By THEODORE S EVANS, M D , AND ROBERT R NESBIT, M D

IT IS FORTUNATE for the orderly and steady progress of medicine that there is, in the profession, a large group of observers slow to accept what is new and unproven, and so it has been true that as each form of leukemia has been described, there has always been a fairly large number of doubting Thomases. This was true when the cleavage between myeloid and lymphoid leukemia was first asserted by Neumann in 1870 and proven by Naegeli and others in 1900. The description by Schilling of a third type of leukemia was greeted by a storm of protest, and the existence of monocytic leukemia is still denied by able and thoughtful observers some thirty-five years after Schilling's original contribution. The existence of basophilic leukemia was first predicated by Joachim in 1906, and more recently studies have appeared by Doan, Groat and others.

Individuals with leukemia showing a marked peripheral eosinophilia have been studied intensively by many workers. In 1912 Stillman described such a case, but many later observers have denied that this was a case of true leukemia. Our own studies indicate that this was probably the first reported case of chronic myeloid leukemia with marked eosinophilic predominance.

We have been impressed in the study of the subject by the following facts:

1. There are comparatively few reports of cases of eosinophilic leukemia, and its occurrence must be quite rare.
2. Many of the reported cases are lacking in essential data as to the maturity of the eosinophils in the peripheral blood, and their descriptions are often inadequate.
3. While many of the case reports include autopsy material, only a small number present evidence regarding the state of the bone marrow during life.
4. In only rare instances have the results of serial bone marrow studies been recorded.

If we accept as a fact that the normal definitive eosinophil is derived from the myeloblast via maturation stages in the bone marrow, then it would seem to be of value to report a case in which studies of both the blood and of the bone marrow showed at first a preponderance of mature eosinophils with later a gradual left shift until finally, myeloblasts replaced a large proportion of the granular cells in both marrow and blood. Hay and Evans, and Thomsen and Plum have reported such instances and have commented upon the value of such information. In our own case of acute eosinophilic leukemia, serial bone marrow examinations and blood films were studied over a period of three months, during which time the gradual change from mature eosinophils through eosinophilic myelocytes to

From the Medical Service of Dr. William Denney and the Pathological Service of the Hospital of St. Raphael, New Haven, Conn.

myeloblasts was evident. While it is impossible to prove that eosinophilic leukemia is a separate and distinct disease entity, paralleling in its life cycle the other well-established types, we believe, however, that the evidence presented adds strongly to previously accumulated data in support of this assumption.

Shapiro in 1919 reported a case of acute eosinophilic leukemia, as did MacDonald and Shaw in 1922, whereas most of the earlier observers referred to their cases as coëxistent eosinophilia and hyperleukocytosis, suggesting that the syndrome may be merely a variant of myeloid leukemia. On the other hand, McGowan and Parker, Stephens, Friedman et al., Thomsen and Plum and others hold that it is a disease entity. At this time there are many proponents of both points of view, and no general agreement has been reached.

### REPORT OF A CASE

A G a 53 year old white female was admitted to the Hospital of St. Raphael\* on June 26, 1946, and died on September 7, 1946.

For approximately five years prior to admission, the patient suffered periodically from itching lumps on the legs. She had been treated with various ointments. These itching subcutaneous lesions would disappear for long periods. In the summer of 1946 they had become fairly widespread and constant and the patient was admitted to the hospital for study. In addition to these itching lumps she complained of recurring attacks of bronchitis and upper respiratory infections which had been noted for many years. The rest of the systemic history was essentially negative. She had lost no strength or weight and slept well except for the pruritis.

Examination revealed an obese, poorly developed woman with rather flabby musculature. The skin was widely and deeply excoriated from scratching so that most of the lesions were almost unrecognizable but a few relatively recent ones were found. The patient stated that they first appeared as lumps beneath the skin which later reached the surface and became red and itchy. Deep-seated masses were found in areas where there was no superficial redness and which did not itch, and other lesions which had reached the surface and had become red and itchy. The masses were rather firm. There was no lymphadenopathy. The moderate fever was assumed to be due to the sepsis from scratching of the skin and consequent infection.

Four days after admission clinical and roentgen ray evidence of broncho-pneumonia was found at both bases. She was treated with penicillin and a favorable response occurred but during the ten weeks in the hospital four similar episodes of fever and lung signs appeared, each of which she survived.

A dermatologist concluded that dermatitis herpetiformis was present. Treatment was ineffective.

A biopsy of the skin lesions gave no evidence of periarthritis nodosa, but mature eosinophilic granulocytes were seen in large numbers, particularly surrounding the blood vessels.

Because of the finding of anemia without any obvious bleeding a hematologic survey was performed early in July. By this time there was some enlargement of the lymph-nodular system and the tip of the spleen could be felt. The peripheral blood showed marked achromia, anis- and poikilocytosis and increased polychromatophilia. Platelets appeared to be present in normal numbers. There was very marked leukocytosis and about 25 per cent of all white cells were adult eosinophils. These were very large, multilobulated and well filled with large granules staining deep red with Wright's stain. Lymphocytes and monocytes appeared to be present in normal numbers proportion and morphology. Sternal puncture showed numbers of active megakaryocytes. There was a marked reduction in the number of nucleated red cells. All stages of myeloid cells were identified from myeloblasts to adult polymorphonuclear cells. The most unusual feature of this marrow was the very large proportion of eosinophils which made up a large part of the total number of white cells.

There was only a slight tendency toward left shift. The eosinophils were very large with polymorphous nuclei. The granules seemed somewhat larger than are usually seen and appeared to be grouped less

\*Service of Dr. William Dennehy

evenly in the cells. The granules also had a tendency to irregular staining with a few cells containing both red and blue staining elements. The average number of granules in each cell was somewhat less than usual. These changes have been described in whole or in part in the cases of Stillman, Shapiro, MacDonald and Shaw, Hay and Evans, and Thomsen and Plum.

Erythropoiesis seemed to have been depressed by the very large concentration of adult eosinophils. The erythro-granulocytic ratio was 1:9.

Tests of the urine, stools, blood nonprotein nitrogen, blood sugar, blood calcium, serology, bleeding, clotting and clot retraction time were normal. The basal metabolic rate was +15 per cent. The erythrocyte sedimentation time was normal. All other causes of eosinophilia appeared to have been eliminated so that the hematologic impression was granulocytic leukemia with marked eosinophilia.

There was a constant eosinophilia during the last eight weeks of life. This varied from time to time but was always beyond normal limits. Serial bone marrow studies showed a steadily increasing tendency toward left shift in the myeloid series. At first many myelocytes, C, were identified in the eosinophilic series. With each succeeding examination of the bone marrow, more B and A eosinophilic myelocytes were seen and finally the bone marrow became strongly blastic in character. The final examination

TABLE 1 — *Blood Counts*

Date	Hg	R B C	W B C	Mature Polys	Bands	Eosinophils		Lymphocytes	Blasts
						Adult	Young		
	%			%	%	%	%	%	%
7-7	55	2,200,000	19,000	50	8	20		22	
7-14	52	2,000,000	21,000	52	10	22	6	26	
7-27	55	2,200,000	13,000	54	9	26	8	20	
7-30	48	2,000,000	30,000					/	
8-6	52	2,000,000	19,000	44	1	27	14	18	10
8-13	44	3,000,000	17,000	40	1	30	19	17	10
8-20	48	2,300,000	14,000	42		39	23	10	5
8-29	48	2,200,000	55,000	30		45	40	1	19
9-6	35	2,000,000	85,000	10		50	5		35

of the bone marrow performed on the day before death revealed a large percentage of myeloblasts with many early (immature) eosinophils.

The patient's condition slowly but definitely worsened with increasing fever, tachycardia, weakness and anorexia. The spleen and liver increased slowly in size and death ensued on September 7, 1946. The clinical diagnosis of leukemia of the myeloblastic type with marked persistent eosinophilia in eosinophilic leukemia was made.

*Postmortem examination* was performed ten and three fourths hours after death. The body was that of a well-developed, moderately obese, middle-aged female. The external body markings of import were marked pallor of the skin and mucous surfaces, superficial ulcerations of the lips, edges of the tongue and buccal mucosa, and a recently incised focus on the left side of the back which appeared to be healing. No evidence of the skin lesions mentioned in the clinical note was seen.

The peritoneal cavity contained no fluid but showed a mass of dense adhesions around the gallbladder. Pleural and pericardial cavities were free of adhesions. Pericardial fluid was normal.

*Heart* 420 Gm. The viscus was markedly pallid. The arteries were slightly thickened but their lumina were patent. Section of the myocardium showed slight gray streaking. The endocardium and valves showed nothing of gross note.

Sections showed a mild degree of infiltration of the epicardial fat by leukemic cells including blast forms, myelocytes and young lobulated forms. Many of these were of the eosinophilic class. The myocardium showed no infiltration and the cells and fibers were normal. There was no fractionating of fibers and striations were normal. The endocardium appeared normal.

*Lungs* Rt. 710 Gm. Left 605 Gm. The two lungs were grossly similar, presenting dark purple red

markedly subcrepitant bases with meaty texture, and very pallid slightly subcrepitant upper lobes. Section revealed markedly increased blood content in the bases, and frothy blood tinged fluid in the remainder, including the smaller bronchi. No foci of consolidation were demonstrable.

Sections revealed an increasingly prominent leukemic infiltration from above downward. Sections of the upper portions showed moderate crowding of the vessels of the alveoli with cells of the leukemic infiltrate, but more marked was filling of the alveolar spaces with precipitated, pink-staining albuminous material in which was a scattering of "heart failure" cells some containing phagocytosed erythrocyte debris, and a few erythrocytes. Sections of the lower lobes revealed intense plugging of the capillaries so much so that the walls of alveoli appeared to be composed of hyperchromatic cells mostly round cell types, which are identifiable as blast and myelocytic forms. These were seen to be just outside of the alveolar epithelium in markedly distended capillaries. The erythrocyte content of these capillaries was practically nil so great was the plugging with the leukemic cells. In many instances there had been rupture of capillaries and adjacent walls of the alveoli so that the alveolar spaces were completely filled with infiltrating leukemic cells and a moderate number of erythrocytes. Large foci were seen that represented confluent ruptured alveoli with air-bubbles in the mass of blast and myelocytic cells. Other fields showed compression of alveolar spaces by the leukemic cells in distended neighboring alveoli.

TABLE 2.—Bone Marrow Differential Counts

Date	Polys	Bands	Myel C	Myel B	Myel A	Blasts	Total Eos	Young Eos	Lym	Mono	Norm	Erythr
7-7	20	25	45	6	2	2	45	15	2	5	9	3

E. M. ratio approximately 1:9. 45% of the nucleated white cells were eosinophils and most of these were adult cells. A few B and A eosinophilic myelocytes were seen.

8-7	15	17	40	14	6	8	45	22				
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45% of the total number of white cells were eosinophils but there was a left shift throughout the myeloid series including the eosinophilic strain.

9-6	12	21	15	13	10	29	46	38				
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On this examination there was seen to be a very marked left shift with the presence of 29% myeloblasts. In addition 38% of the eosinophils were myelocytes and there were some cells in which both blue and red staining granules are identified. Many cells were seen in mitosis.

*Spleen.* 1235 Gm. This organ was greatly enlarged and its capsule tense and ironed out but the consistency was of a tensely fluctuant nature rather than hard. When sectioned the cut surface everted and rolled the stretched capsule back. The cut surface was predominantly gray in color and the pulp, greatly increased, was essentially of gray color. To touch this surface was greasy.

Sections revealed sinusoids packed with leukemic cells of the type already described and large foci of acute necrosis some of which could be seen to be splenic follicles. These foci contained neutrophilic polymorphonuclears and macrophages, in a mass of necrotic cells. Some other foci showed replacement fibrosis of these necrotic islands this of varied age some partly hyalinized others with young fibroblasts proliferating in foci still showing some of the acute process. Besides the leukemic cells, there were, in the sinusoids scatterings of macrophages containing engulfed cell fragments, and pigment. The endothelial lining cells were not remarkable except that they were generally flattened by the widely distended sinusoids.

*Pancreas.* 85 Gm. Grossly, the pancreas showed normal surface and section markings, and its duct appeared normal.

Sections showed infiltration essentially peribiliary, and in only rare instances was there any infiltration from the peribiliary connective tissues into the gland itself. The capillaries contained the leukemic cells in large numbers, but they were noticeably absent from the parenchyma of the gland. The islet and alveolar cells appeared normal and retained normal staining reactions.

TABLE 3 — *Reported Cases of Eosinophilic Leukemia*

Author	Age	Duration	Range of W B C.	Range of eosins	Type eosins	Organs affected
<i>Acute Cases</i>						
Hay and Evans 1929 Case 1	41	3 weeks	72,000*	83*	Mature	Lymph nodes, Spleen
McCowan and Parker 1932	45	2	154,000-20,000	78	Myelocytes, 30%	Spleen
Stephens 1935	17	10	130,000	68	Metamyelocytes 1%	Lymph Nodes Lungs
Forkner et al 1937	33	4	265,000-118,000	82-75	Many Myeloblasts	Lymph Nodes, Spleen Liver, Lungs
Ravault et al	60	12	28,000-71,000	80-90	Myelocytes, 30%	Lymph Nodes, Spleen Liver Many others
<i>Chronic Cases</i>						
Stullman 1912	27	Un known	165,000-118,000	91-85	Metamyelocytes, 20%	Liver, Lymph Nodes, Spleen
Griffin 1919	31	7	211,000-15,700	90-75	Mature	Lymph Nodes, Spleen, Liver Heart
Shapiro 1919	48	6	236,000-15,000	90-49	Myelocytes, 5%	Lymph Nodes, Spleen Liver Lungs
McDonald and Shaw 1922	46	8	138,000-34,000	82-71	Mature	Lymph Nodes Spleen
Alexander 1924	50	9	150,000-17,000	36-21	Mature	Spleen Liver Lungs Heart
Hay and Evans 1929 Case 2	53	3	62,000-16,000	55-16	Mature	Spleen Liver Heart, Kidneys
Drennan and Biggart 1930	15	2	73,000-32,000	70-23	Mature	Lymph Nodes Spleen Liver Lungs L. Leg
Harrison 1930	23	2	16,000-13,000	60-30	Mature	Lymph Nodes Liver Lungs Spleen
Thomsen and Plum 1939	11	1	65,000-4,000	90	62% Mature Later 81% Myeloblasts	Lymph Nodes Liver Spleen Heart
Gochl 1941	18	1	190,000-8,000	88-5	Mature	Lymph Nodes Spleen
Friedman et al 1944	11	½	124,000-194,000	80-90	Mature	Lymph Nodes Liver Spleen Many others
Ravault et al 1944	60	½	28,000-71,000	63-78	30% Immature	Lymph Nodes Liver Spleen
Hodgson et al 1945	44		47,000	45	Mature	Lymph Nodes Liver Spleen Many others

\* Terminal





FIG. 1.—Liver section showing the intense and essentially portal infiltration by the leukemic cells the resulting fatty degeneration also pyknotic nuclei more centrally, and the fading into parenchymatous degeneration near the central vein which is seen to contain large numbers of hyperchromatic cells with large nuclei the leukemic cells. Phloxine hematoxylin  $\times 450$ , enlarged from 35 mm film.

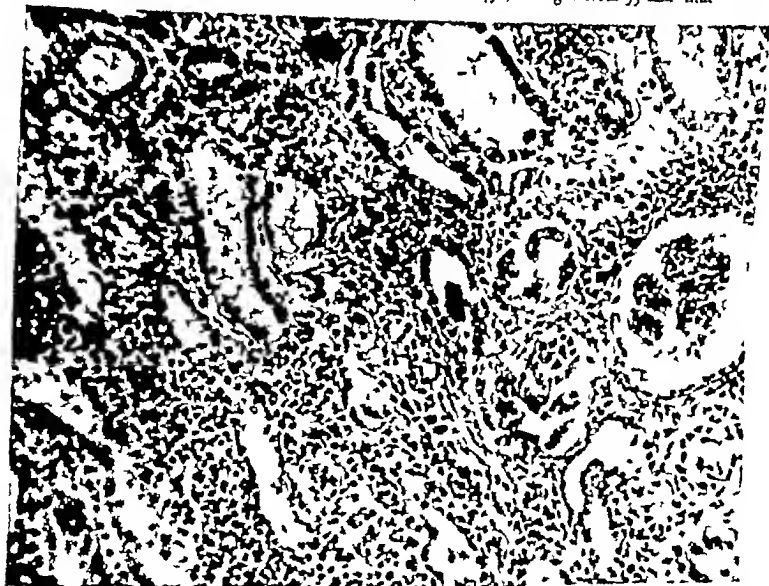


FIG. 2.—Kidney section, showing intense infiltration of the interstitial tissues and at right the concentration of the infiltrating cells in the capillary about Bowman's capsule. The appearance of a round cell stroma is typical of all sections. Phloxine hematoxylin stain enlarged from 35 mm using  $\times 450$ .

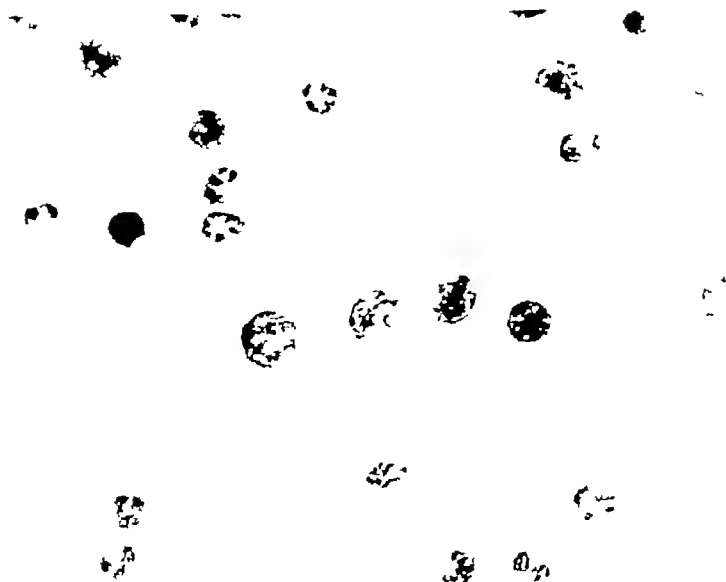


FIG 3 —Smear of first bone marrow aspiration showing mature polynuclear neutrophils eosinophiles and late myelocytes

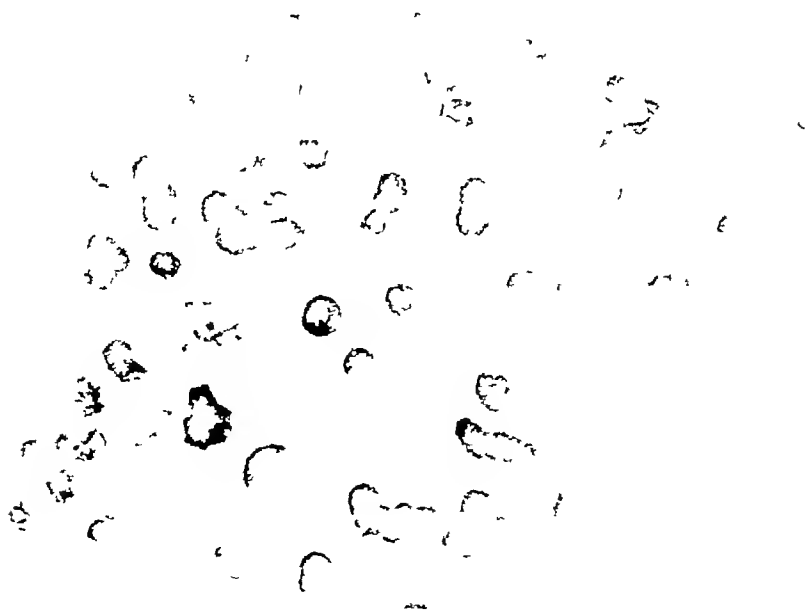


FIG 4 —Smear of third bone marrow aspiration showing many young cells and oo mature polynuclears

*Gastro-intestinal tract* No gross abnormalities were noted. There were no foci of hemorrhage and no ulcerations. The lymphoid patches showed some swelling, but no necrosis.

*Liver* 1625 Gm. There was marked enlargement of the liver, which had a rounded edge, and a pale mixed yellowish brown and white color both on its capsular and cut surfaces. The cut surface bulged prominently and felt greasy. Markings were almost obliterated. No gross abnormalities of the biliary system were demonstrable.

Microscopically, there were two notable processes both most marked in the portal zones of the lobules and fading as the central zones were reached. These were an intense leukemic infiltration of the entire portal region, and a likewise intensely marked fatty degeneration of the hepatic cells. This was combined with moderate crowding of the sinusoids with the leukemic cells though the latter was much less prominent than the portal infiltration. The cells of the liver cords were in varied stages of necrosis as well as fatty degeneration and pyknotic nuclei and disintegrating cytoplasm were common. In the midst of the leukemic process, the bile capillaries stood out and appeared remarkably unaltered. No cirrhotic process was seen and there was no bile duct proliferation. The vessels of the portal region all contained an excess of the leukemic cells as did the central veins. The cells nearest the central veins showed some parenchymatous degeneration but were comparatively well preserved.

*Gallbladder* In the ampullary region of the cavity was a partially impacted calculus, 1 cm. in diameter, composed of concentric layers of cholesterol and pigment about a pigment nucleus.

*Adrenals* Rt. 7 Gm. L. 6 Gm. Aside from marked autolysis of the medullae the glands were not grossly remarkable.

Sections showed very mild spotty infiltration in cortical and medullary zones. Aside from this and marked autolysis of the medullary cells, the glands were essentially normal.

*Kidneys* Rt. 400 Gm., L. 425 Gm. These two organs were similar in gross. They were large and pale. The capsules were free and stripped with ease. The external and cut surfaces were pallid and mottled, and the cut surfaces greasy. Markings were largely obliterated, but the cortico-medullary ratio was retained, though both were greatly increased in width.

Sections showed an intense infiltration of the interstitial tissues by leukemic cells. Of especial note was an intensely marked infiltration of the pericapsular region apparently in the capillaries with no similar distention of the capillaries of the glomerular tufts. The glomeruli stood out normally in the round cell background with normal appearing subcapsular spaces surrounding them and normal Bowman's capsules around the whole. The tubules all classes showed prominent parenchymatous degeneration. The interstitial tissues were so completely infiltrated that in many instances normal tissues were invisible or appeared fragmentarily in small foci. The infiltration extended to the pelvis and calices, where it was seen just beneath the epithelium, the latter appearing normal.

*Lymph nodes* All nodes were greatly enlarged, gray white in color and tense but not hard. Their cut surfaces bulged prominently and were gray and greasy to sight and touch.

Sections revealed a process similar to that in the spleen with the exception of necrosis. Sinusoids were crowded with the leukemic cells and the architecture of the nodes was destroyed by its intensity. The follicles were practically absent and the nodes largely replaced by the leukemic infiltrate.

*The bone marrow* was abundant and almost white but sections were not satisfactory, probably because of the long postmortem period before removal.

A clot in the pulmonary artery was used for Wright's staining and demonstrated the leukemic cells amply. The cells were essentially of the eosinophilic classes of myelocytes with numerous blasts present and a moderate number of mature forms in all classes. Of the mature forms most were eosinophils and the remainder were neutrophils in their staining reaction. A few lymphocytes were seen.

The essential findings were leukemic in origin being most marked in the liver, spleen, kidneys, lymph nodes and lungs. Other changes such as those of fatty degeneration of the liver were secondary to the process. Especially notable were the massive infiltrations of the lungs the essentially portal infiltration of the liver, and the interstitial infiltration of the kidneys. The collection of the infiltrating cells in the pericapsular capillaries of the glomeruli was outstandingly prominent.

#### COMMENT

A complete search of some of the more recent literature has been impossible, since many of the foreign journals are not yet available. Only a few cases of eosino-

philic leukemia have been studied by means of both serial bone marrow spreads and postmortem material. The marrow studies in this case showed a progressive development of the leukemic process. In the earlier study, most of the eosinophils were adult cells. Gradually there was replacement of these mature granulocytes by younger forms (early eosinophilic myelocytes) and eventually a shift to blast cells. The early myelocytes contained both red and blue staining granules within the same cell. These have been noted before by MacDonald and Shaw, Hay and Evans in eosinophilic leukemia and by Doan and Reinhart in basophilic leukemia and have been considered to be evidence of left shift. Additional evidence of left shift is seen in the presence of mitotic figures. The increasing number of young marrow cells appeared largely in the eosinophilic strain—from 15 per cent in the first study to 22 per cent and finally to 38 per cent. The same sequence of events was seen to a lesser extent in the peripheral blood where blasts to the number of 20 per cent appeared at one time. The finding of 45 per cent eosinophils in the peripheral blood of which 30 per cent were young eosinophil granule cells was made on one occasion.

The separation of eosinophilic leukemia from many other conditions which result in secondary eosinophilia in the peripheral blood is always difficult. Stewart has reviewed the literature on familial eosinophilia and Paviot and others, that on eosinophilia associated with malignant disease. Henschen has written a comprehensive review of the whole subject. Reports of eosinophilia with recurrent attacks of lung infiltration (Loeffler's syndrome) are becoming increasingly frequent in the literature. Although our patient had several attacks of pneumonia during the period of observation, there were too many clinical facts at variance with this condition, and the autopsy findings were too definitely conclusive of leukemia to place our case in that category. Periarteritis nodosa was also considered in the differential diagnosis of this case, but skin biopsy was negative for this condition, and postmortem examination did not support this diagnosis.

The diagnosis of eosinophilic leukemia may be initially and tentatively advanced on the persistent presence of a large percentage of eosinophils in the circulating blood. If a considerable and increasing number of these cells are eosinophilic myelocytes, reflecting predominance in the bone marrow, the evidence is still further supportive, and if the disease is fatal and there is invasion of all the organs by these abnormal cells as shown at postmortem examination, the diagnosis may be said to have been established. All of these criteria were present in our case, including the observation of the progressive left shift in the eosinophil granule myelocyte to the myeloblast which predominated terminally.

Doan and Reinhart have reported the presence of an acute dysfunction of the bone marrow, resulting in fulminating basophilic leukemia. They have called attention to the fact that immature elements of the different cell strains may be present at the same time in the blood of a given patient. They have also noted the fact that both basophilic and eosinophilic granules were found in the same cell in basophilic granular cell leukemia but that the basophilic granules were present in larger proportion. Our case is similar in that both types of granules were present in individual cells but differs from theirs in that eosinophilic granules were preponderant. A further parallel to their cases is seen in the fact that our case also

showed a left shift to the primitive cells, however, our case progressed to the blastic phase through eosinophilic granule myelocytes, whereas theirs reached the ultimate state of myeloblastosis through basophilic granule myelocytes. Doan and Reinhart conclude that there is an initial benign, perhaps metabolic disturbance in the granulopoietic equilibrium,—in the normal reciprocal relationships which seem to characterize the body cells in health,—to be followed sooner or later, especially in the later decades of life, by a very differently acting, invasive, metastasizing process much more closely related in clinical course and cellular pathology to the malignant hyperplasia and anaplasia which characterize tumor growths arising in other organs.

The total and proportional number of eosinophil granule cells in our case did not reach the extremely large numbers reported by some other observers, but it should be pointed out in this connection that this patient died in the acute stage of the disease. Death in other types of leukemia often occurs with low peripheral blood counts but with all other evidences of leukemia, and it has been assumed that this is so because the disease is fatal before a massive cellular response is seen in the blood.

#### SUMMARY

1 The data in a case of fatal leukemia with predominant eosinophilia in the peripheral blood and bone marrow are presented, we believe that this case was one of eosinophilic leukemia.

2 During the period of observation, these eosinophils showed progressive immaturity as the symptoms became more severe. Eventually this left shift became so marked that a large proportion of the cells were terminally myeloblasts in both the blood and the bone marrow.

3 Autopsy revealed invasion of many of the tissues and organs with these mature and immature eosinophil granulocytes and with myeloblasts.

#### ACKNOWLEDGMENT

Our thanks for helpful criticism are particularly extended to Dr. Charles Doan and to Dr. William Dameshek.

#### REFERENCES

- 1 ALEXANDER, M. E. Observations on a case of splenomegaly with hyperleukocytosis. *J. Lab. & Clin. Med.* 9: 803, 1924.
- 2 ARMAND DE LILLE, M. D. ET AL. Hyperleucocytose avec éosinophilie. *Bull. soc. de pédiat. de Paris* 25: 424, 1927.
- 3 AUBERTIN, C. AND GIROUX, L. Existe-il une leucémie à éosinophiles? *Presse med.* 29: 314, Part 1, 1921.
- 4 BASS, M. H. Unusual case of eosinophilia with splenomegaly. *Am. J. M. Sc.* 179: 416, 1925.
- 5 ——— Eosinophilic leukemia in a child. *Am. J. Dis. Child.* 41: 1394, 1931.
- 6 ——— Eosinophilic leukemia. *Am. J. Dis. Child.* 62: 68, 1941.
- 7 BEIFELD, A., AND BARNES, M. Severe anemia with eosinophilia. *Bull. Johns Hopkins Hosp.* 27: 181, 1916.
- 8 DOAN, C., AND REINHART, H. The basophil granulocyte basophilcytosis, and myeloid leukemia basophil and mixed granule types: an experimental clinical and pathological study with the report of a new syndrome. *Am. J. Clin. Path.* 11: 1, 1941.

- <sup>9</sup> DOAN, C. Personal communication
- <sup>10</sup> DOWNNEY, HAL. Handbook of Hematology New York Paul Hoeber 1938 1 178 and 4 2949
- <sup>11</sup> FORENER, C. ET AL. Eosinophilic myelogenous leukemia Chinese M J 51 609 1937
- <sup>12</sup> FRIEDMAN, M., ET AL. Eosinophilic leukemia report of a case Am J M Sc 208 333, 1944
- <sup>13</sup> GIFFIN, H. Persistent eosinophilia with hyperleukocytosis and splenomegaly Am J M Sc 158 618 1919
- <sup>14</sup> GOEHL, R. Eosinophilic leukemia with a case report Journal Lancet 62 252 1942
- <sup>15</sup> GROAT, W. ET AL. Acute basophilic leukemia Am J M Sc 191 457 1936
- <sup>16</sup> HARRISON, F. Eosinophilia with splenomegaly Am J M Sc 170 208 1930
- <sup>17</sup> HAY, J. AND EVANS, W. Acute eosinophilic leukemia and eosinophilic erythro-leukemia Quart J Med 22 167 1929
- <sup>18</sup> HENSCHEN, C. Über hochziffrige Eosinoleukaemien Deutsch Ztsch f Chir 243 1, 1934
- <sup>19</sup> HODGSON, J. ET AL. Myeloid leukemia probably eosinophilic in character Am J Clin Path 15 389 1945
- <sup>20</sup> JACOBSEN, M. Case of leukemia and eosinophilia Act med Scand 119 192, 1944
- <sup>21</sup> LEPAK, J. Eosinophilic leukemia with discussion of the literature Minnesota Med 23 596 1940
- <sup>22</sup> McCOWAN, G. ET AL. Case of acute eosinophilic leukemia J Roy Nav Serv 18 131, 1932.
- <sup>23</sup> McDONALD, S. AND SHAW, A. Persistent eosinophilia with splenomegaly Brit M J Part 2, 966 1922.
- <sup>24</sup> PAVIOT, J., ET AL. L'éosinophilie sanguine des tumeurs malignes (à propos d'un cas de reticulosarcome péri-renal avec éosinophilie sanguine et tumorale) Ann d anat path 12 113, 1935
- <sup>25</sup> PRAVAIL, P. ET AL. La leucémie aigue à éosinophiles Sang 16 258 1945
- <sup>26</sup> ———. Leucémie à éosinophiles poussée aigue terminale Étude critique des éosinophiles essentielles et de la leucémie aigue à éosinophiles Lyon med 168 57 1942.
- <sup>27</sup> REYE, D. Case of eosinophilic leukemia M J Australia 2 156 1940
- <sup>28</sup> SCHMIDT, WETLAND, P. Eosinophilic leucemoid Med Klin 21 1767, 1925
- <sup>29</sup> SEEMAN, G., AND SAJZEWA, A. Ein Fall von Chloromycelose mit ungewöhnliches Eosinophilie Fol Haemat 37 258 1918
- <sup>30</sup> SHAPIRO, L. Eosinophilic leukemia report of a case Proc N Y Path Soc. 19 73 1919
- <sup>31</sup> STEPHENS, D. J. Acute eosinophilic leukemia Am J M Sc. 189 387 1935
- <sup>32</sup> STEWART, S. Eosinophilic hyperleukocytosis in Hodgkin's disease Arch Int Med 44 772 1929
- <sup>33</sup> STILLMAN, R. Myeloid leukemia with predominance of eosinophilic cells Med Rec 81 594 1912.
- <sup>34</sup> SVANBERG, T. Eosinophil leukocytosis and peri arteritis nodosa Nord Med (Hygeia) 22 948 1945
- <sup>35</sup> TECOZ, R. M. ET AL. Éosinophilic leucocytose éosinophilique éosinophilique pseudo-leucémie Helv med acta 10 17 1943
- <sup>36</sup> THOMSEN, S., AND PLUM, P. Eosinophilic leukemia Acta Med Scand 101 116 1939
- <sup>37</sup> ———, AND ———. Eosinophilic leukemia Ugesk. f Laeger 101 90 1939
- <sup>38</sup> TISCHENDORF, W. Zur Frage eines besonderen Krankheitsbildes mit Eosinophilie Ztschr f klin Med 137 787 1941
- <sup>39</sup> VALLEDOR, T. ET AL. Síndrome leucemoide eosinofílico Bol soc cubana de pediat 11 207 1939
- <sup>40</sup> ———. El síndrome leucemoide eosinofílico Bol soc cubana de p-diat. 14 18 1942.
- <sup>41</sup> ———. La eosinofilia masiva a tipo leucemoide de origen infeccioso y evolucion regresiva, comouna nueva Bol soc cubana de pediat 15 923 1944
- <sup>42</sup> WISEMAN, B. Personal communication

# MEGAKARYOCYTES IN NORMAL AND IN THROMBOCYTOPENIC INDIVIDUALS

## WITH INTRODUCTION OF A NEW SYSTEM OF DIFFERENTIAL COUNT FOR MEGAKARYOCYTES

By VICTORINO DE LA FUENTE, M D

OF THE THREE principal cellular elements of the bone marrow, the megakaryocytes\* have been least investigated. This paper deals with them as found in (1) hematologically normal individuals and in (2) thrombocytopenic patients. It is the first in a series of papers given to the study of the participation of these cells in various pathologic states.

### THE NORMAL MEGAKARYOCYTE AND ITS DEVELOPMENT

#### *Materials*

The subjects of the present project are human—some normal and others abnormal from the hematologic standpoint.

The statement made below on the normal line of development of the megakaryocytes is an integration of all the observations made on the bone marrow specimens of these individuals.

#### *Methods*

The bone marrow specimens were secured in the usual way by sternal puncture. The amount obtained did not exceed 0.5 cubic centimeters. Without using an anticoagulant, the material was spread on slides as in preparing blood films. Seven to ten smears were made. The smears were made thin, slightly thick and thick. The thick ones were used to obtain a general impression of the condition of the bone marrow at the time of the biopsy. For a differential count of the megakaryocytes, those films were examined which showed a great number of these cells without morphologic distortion. It has been observed that these smears could not be used with satisfaction for the differential examination of the smaller bone marrow cells.

Before sternal puncture was made, the total platelet count in the peripheral blood was determined. Either the wet smear method of Dameshek (D) or the dry method of Fono (F) was employed, depending upon convenience. Other hematologic examinations were carried out to determine normality, or for the sake of diagnosis.

The smears were fixed with Wright's solution and stained with Giemsa's.

The number of megakaryocytes per million nucleated cells was not counted for two reasons: (1) Even in a carefully made smear the megakaryocytes, being heavier than other types of cells, tend to gather more at the initial portion. Hence, more megakaryocytes are to be found in this area than in any other portion of the smear. (2) Since the value is relative and since no method so far is accepted as reliable in determining the absolute number of nucleated cells per unit volume of the bone marrow material, the

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From the Department of Internal Medicine, University of Santo Tomas, and the Clinica Sugrao, Manila, Philippines.

Supported by a Grant from the Division of Research Grants and Fellowships, National Institute of Health, U. S. Public Health Service.

\*Due to lack of a generic term embracing all types of megakaryocytes, the latter is used in this paper in two senses: in general, to designate the series in contrast with the myeloid and erythroid series; and in particular, to designate the mature type in contrast with the megakaryoblasts and promegakaryocytes. In what sense it is used in a given sentence may be inferred from the context. When the latter is not clear, a modifier is employed.

result does not justify the time and energy consumed in the procedure. A rough calculation made by an experienced examiner would be sufficient for clinical purposes. One can make a fairly reliable judgment of the general condition of the megakaryocytic tissue from the number of smears used to finish a differential count of 100 cells. With the method used in this project, from one to two smears had to be examined to cover 100 cells when the megakaryocytes were not disturbed. In idiopathic thrombocytopenic purpura, only about one third to one half of a smear was enough.

In order to speed up the examination, the low power objective was used to spot and bring the megakaryocytes within the microscopic field. The cells were then examined under the oil immersion objective for structural details. With this procedure, it is possible to go over the same area twice. To avoid this mistake, thin lines perpendicular to the length of the slide were drawn at various intervals. If the smear is examined under the low power objective beforehand, lines can be drawn on areas free from megakaryocytes.

All the photomicrographs were taken under the same magnification using a 6X ocular. Actual magnification cannot be calculated for lack of equipment. The size of each cell may be estimated by comparing it with the lymphocyte and erythrocytes in figure 15. The description of each picture is incorporated in the text.

### *Origin and Normal Development of the Megakaryocytes*

The origin of the megakaryocyte is largely controversial. It has been traced to almost all types of blood cells and data are at hand to support each theory. Nevertheless, proofs to establish one hypothesis are not so conclusive as to exclude the others.

A better evidence is present with regard to the normal line of development followed by these cells as they mature and give rise to platelets. Knowledge of this is important for clinical purposes, particularly in the classification of cells.

The mode of development had been treated extensively in a previous paper.<sup>1</sup> Additional light has been shed by later observations.

The existence of the megakaryoblast is an indisputable fact. With the low power objective it can be easily distinguished in the smear. It is bigger than other blast cells commonly found in the bone marrow (figs. 1-8).

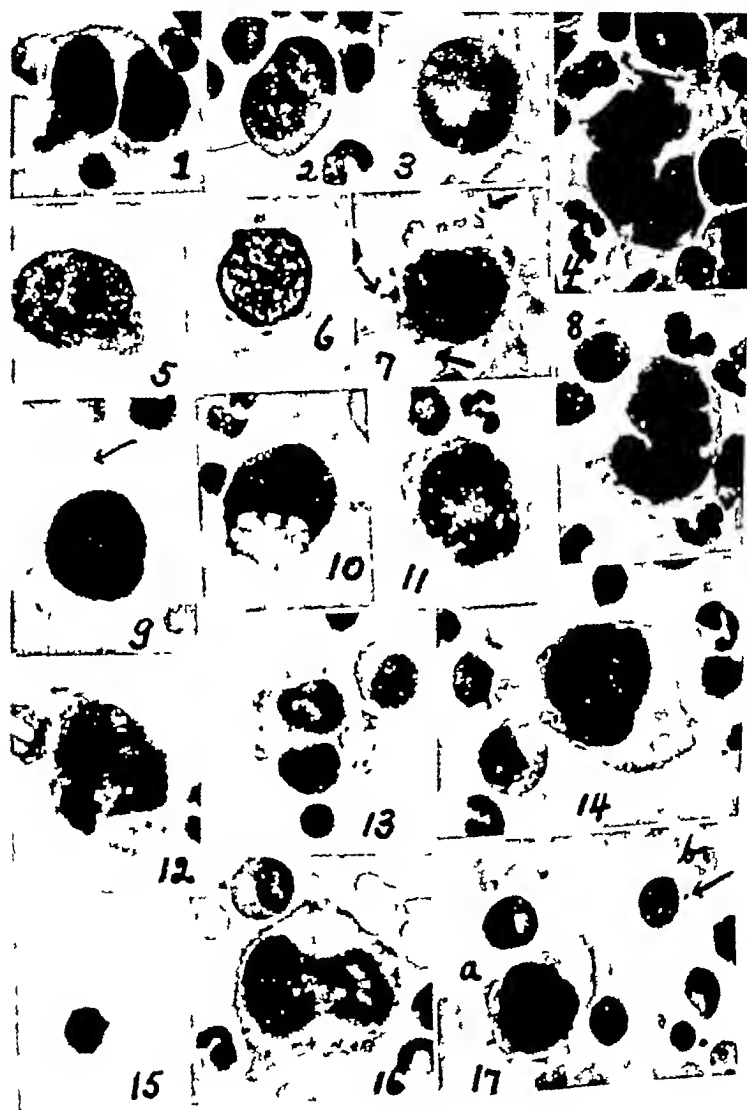
The preferred mode of multiplication is amitosis. This is supported by the constant increase of amitotic forms (fig. 1) when the total megakaryocytes are increased. That such forms belong to the megakaryocytic series is inferred from the presence, in some instances, of platelets in their cytoplasm (fig. 13). Mitosis occurs rarely.

The maturation usually starts in one of the nucleoli. The latter widens and becomes more transparent (figs. 11 and 12). The process continues towards the margin of the nucleus, which subsequently assumes a lobulated figure (fig. 18). When nucleoli near the nuclear margin undergo the same change, the nucleus presents a rugged and eroded edge with granules strewn in the neighborhood (fig. 10). The presence of a round or oval nucleus in the promegakaryocytic phase (fig. 9) would seem to indicate that after transformation of a portion of the nuclear chromatin into granules, the nucleus may assume a round contour. Forms in the mature stage are seen whose nuclei are reduced to small specks, at times darkly staining, embedded in or surrounded by a mass of granules (figs. 19 and 24).

The origin of the cytoplasmic granules from the nucleus has already been mentioned by Downey.<sup>2</sup>



The granules upon dispersal throughout the cytoplasm may be scattered evenly or may congregate into groups with subsequent formation and fragmentation of



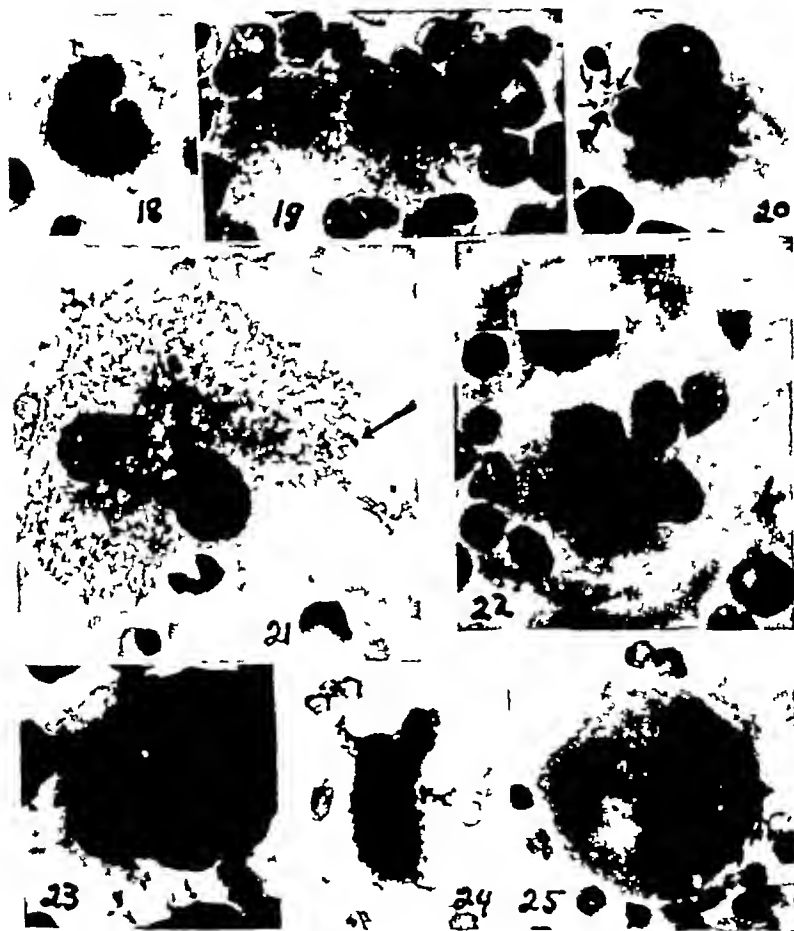
FIGS. 1-17—See text

platelets. At times when formation of normal platelets is disturbed and the peripheral blood is thrombocytopenic, the cytoplasm divides into big shreds.

The so-called blue platelets, which are really fragments of a basophilic cytoplasm, are not peculiar to the megakaryocytes alone. Lymphocytes in the blood

may exhibit the phenomenon too (fig. 15). The same is true of the immature eosinophiles and neutrophils in the bone marrow.

I have expressed the opinion before<sup>1</sup> that the polykaryocyte was an indication of aborted amitosis. This appears to be borne out by subsequent observations. A binuclear or multinuclear mature megakaryocyte may be the resulting form of two



FIGS. 18-25 — See text

processes. It may be the subsequent phase of an amitotic megakaryoblast whose cytoplasm fails to divide. It therefore passes through the promegakaryocytic stage (fig. 17a). Or it may be the result of a complete division of the nucleus of a mature form, in the same manner as may be observed sometimes in a segmented neutrophil. In figure 21, the two lobes are connected by a barely visible filament. In figure 22, the thin bridge between one lobe and the rest of the nucleus is very clear. It is not

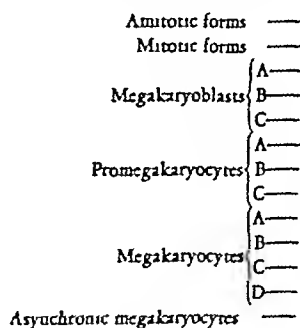
far-fetched to suppose that after severance, the lobe would assume an oval shape. The appearance of two lobes connected by a thin thread of chromatin does not argue for the fusion theory. In case of fusion, it is likely that the two nuclei would approach each other tangentially.

### *Classification of the Megakaryocytes*

The differential count is an important tool in the hands of the hematologist. A good system of differential counting, one which reflects adequately and reliably the reaction of the cells to pathologic stimuli, is an invaluable aid to diagnosis, prognosis and evaluation of treatment. This is shown in the Schilling hemogram and Price-Jones curve, which basically is a grouping of erythrocytes according to diameter.

Pathologic states usually alter the process of cellular maturation. Thus, in classifying cells for clinical purposes, this fact should be taken into consideration. Similarly, whenever possible the state of functional activity should also be reflected in the differential count.

We have borne these two things in mind in proposing the following classification of the megakaryocytes. The latter are divided into



*Explanation.* The major division (amitotic forms, etc.) gives an insight into the multiplication and maturation of the cells at the time of aspiration. The subdivision of cells into A, B, etc., measures the physiologic activity of the megakaryocytes with respect to the production of platelets and remotely to the cessation of bleeding.

The megakaryoblast (figs 2-8), promegakaryocyte (figs 9-14, 16, 17a, and 18) and megakaryocyte (figs 19-22 and 24-26) need not be described in detail. Their description found in literature, especially in the article of Dameshek and Miller,<sup>3</sup> leaves nothing to be desired and is adhered to in this paper.

The asynchronous megakaryocyte is one whose nucleus belongs to one stage and whose cytoplasm to another. Unlike the granulocytes, the correlation between the nuclear and cytoplasmic development in the megakaryocytes is not so close. Hence, allowance should be made on this account. Yet a distinct type of megakaryocyte may be isolated from the rest, characterized by a nucleus frankly mature and a cytoplasm that is basophilic, without any trace of granularity and without platelets.

formed. In other words, the development between the nucleus and cytoplasm is separated by one phase, that is, *asynchronous*. This type of cell is seen even in normal individuals. Figure 23 illustrates it. The platelet-like bodies at the lower portion of the cytoplasm are artefacts.

The subdivision of the megakaryocytes into A, B, etc, cells is based on the presence of platelets near or at the margin of the cytoplasm. Platelets found near the nucleus and far from the edge of the cytoplasm are not immediately available to the peripheral circulation. Hence, they are not taken into account in the sub-grouping of the cells from the functional standpoint.

A cells do not exhibit platelets in the cytoplasm, or if they do, the platelets are centrally located. Megakaryoblast A and promegakaryocyte A, except in rare instances (fig. 9), offer no difficulty, for these cells have very scanty cytoplasm so that platelets found in them are always near or at the margin. It is in the mature megakaryocyte that the location of the platelets should be scrutinized. Hence, under megakaryocytes A, mature cells without platelets and those whose platelets are far from the cytoplasmic margin are grouped together. (Figures 2-6 are megakaryoblasts A, figures 9-11, 14, 16 and 17a are promegakaryocytes A. In figure 9, arrow points to a lone platelet well inside the cytoplasm. Figure 19 is a megakaryocyte A. The cytoplasm of this cell is granular. The isolated platelet-like bodies are artefacts.)

B cells are those whose cytoplasm contains one to ten marginal or juxta-marginal platelets. Number 10 has been chosen as the limit, because in the acute form or phase of Werlhof's disease, not a single megakaryocyte with more than ten platelets had been found. On the contrary, the B' cells may be as numerous as in the normal. (Figure 7 is a megakaryoblast B, figure 22a is a promegakaryocyte B, figures 17b, 20 and 21 are megakaryocytes B. Each arrow points to a platelet. Other similar bodies are artefacts.)

C cells are those whose cytoplasm contains more than ten marginal or juxta-marginal platelets. (Figure 8 is a megakaryoblast C. This cell is not found normally and is rare even in pathologic cases. Figures 12, 13 and 18 are promegakaryocytes C. Figures 22b, 24 and 25 are megakaryocytes C. Figure 25 illustrates a nucleus in process of gradual dissolution.)

D cells are those whose cytoplasm is wholly converted into platelets (fig. 26). Polykaryocytes (figs. 13, 17a and 17b) are considered frustrated amitotic forms. They are grouped under either the promegakaryocyte or megakaryocyte, depending upon the developmental stage reached, and into A, B or C cells depending upon the number of platelets present.

In introducing a new classification of cells according to maturity, the greatest difficulty met by the proponent lies in the imparting to his readers, who may wish to investigate its value, the different characteristics found in a cell so that the latter may be placed in the group to which it should belong. The problem is succinctly summed up in the question: At what point does the megakaryoblastic phase end and the promegakaryocytic begin?

In this classification, the cell is considered as a whole. The morphology of the nucleus receives the first attention. When for some reason or other the nucleus

does not provide a clear-cut basis for deciding the age of the cell, the cytoplasm becomes the determining factor

Under the amitotic forms, only those with blastic nuclei are included, because when a multinucleated megakaryocyte enters the promegakaryocytic stage, its cytoplasm appears to be no longer capable of division. A cell is classified as amitotic only when the two or more daughter-nuclei are clearly separated by a portion of the cytoplasm (fig. 1)

In the bone marrow there are other cells which seem to be undergoing amitosis. Most of them are syncytial erythroblasts. However, the amitotic forms of the megakaryocytic series are much bigger than the erythroblasts. When in doubt, nevertheless, it is best not to include the cell in the differential count



FIG. 26—See text. A greater magnification than figures 1-25

A megakaryoblast has commonly a nucleus exhibiting nucleoli (figs. 2, 3, 4, 5, 7 and 8). Sometimes the nucleus is in the initial stage of dividing into several daughter-nuclei (figs. 2, 3, 4 and 8). It has been said that the megakaryoblast does not produce platelets. However, figures are observed with distinct nucleoli in the nucleus and platelets in the cytoplasm (figs. 7 and 8). Megakaryoblast B is not uncommonly observed even in normal individuals. Megakaryoblast C is rare and was seen by the writer only on one occasion.

In some instances, a megakaryoblast is encountered without distinct nucleoli (fig. 6). However, the size would not permit its inclusion into the erythroid or myeloid group. Furthermore, its nuclear chromatin materials are disposed in coarser strands than those of the myeloblast. The cell would appear to be what Downey, Palmer and Powell<sup>4</sup> consider a transitional form from the myeloblast to the megakaryoblast by a process of swelling.

The cell has entered the promegakaryocytic phase when the definite structure of the nucleoli is lost, especially when a transparent zone takes the place of one of the nucleoli (figs. 11 and 12). In the meantime, granules appear. A young promega-

karyocyte may also present an eroded nuclear margin (fig 10) with granules replacing the dissolved nuclear substance. The nucleus prior to entrance into the fully matured state may present a hazy appearance.

There are times when the morphology of the nucleus does not help at all in the classification of the cell. The former looks like one massive dark spot. It may either be a promegakaryocyte or a megakaryocyte, depending upon the cytoplasm. A cytoplasm partly basophilic would cause the cell to be included among the promegakaryocytes, a cytoplasm evenly studded with fine lilac granules among the megakaryocytes. Figure 24 illustrates this variety of cell. The nucleus, situated at the lower pole of the cell, could hardly be made out. The other dark spots consist of intensely staining granules massed together. With the use of a 6X ocular the different portions of the cell may be distinguished.

In the subdivision of the cells into A, B, etc. cells, very little difficulty is encountered. When there is doubt as to whether a structure in the cytoplasm is a platelet or not, it is best to refer to the morphology of isolated platelets in the same bone marrow film or in the smear of the same patient's blood.

It should be stated that the proposed classification may possibly suffer from one defect. It is likely that a given megakaryocyte, which had already shed a large number of platelets into the peripheral circulation, may appear as an A cell. In order to establish or rule out the presence of such an error in a given case, the platelets and their disposition should be examined. In normal and in increased production of platelets, the latter are clustered together in big sheets. In decreased production, very few are seen and they are distributed singly.

Table 1 gives the differential counts of the megakaryocytes in 5 hematologically normal individuals. The patients were from the Eye, Ear, Nose and Throat Ward of the Santo Tomas University Charity Hospital, confined for such troubles as strabismus and other local eye disorders which are known not to affect the general system. Their peripheral blood had been examined and all values were within the normal range.

#### THE MEGAKARYOCYTES IN THROMBOCYTOPENIAS

After Wright had demonstrated the derivation of platelets from fragmenting megakaryocytic cytoplasm, it was logically expected that the origin of thrombocytopenia would be tracked down to a disturbance of this bone marrow element. Except for rare cases, a low platelet count is generally associated with a disturbed megakaryocytic picture.

The following cases of thrombocytopenia of varying etiology exemplify the different types of megakaryocytic response to pathologic stimuli.

#### *Materials*

Twenty cases suffering from different diseases in which thrombocytopenia was present are reported. The megakaryocytic reactions (tables 2, 3 and 4) had been so uniform that they could be separated and classified into three types. The cases are therefore grouped accordingly. The important findings in each case are summarized in tables 2a, 3a and 4a.

*Group I* (table 2a) includes only cases of idiopathic thrombocytopenic purpura. No attempt is made to subdivide the disease into acute and chronic. On the basis of

TABLE 1.—*Differential Counts (in %) of Megakaryocytes in Normal Individuals*

Cells	D B (f)	P C. (m)	M P (f)	F S (m)	B Y (f)	Range
Amitotic forms	1	0	1	0	0	0-1
Megakaryoblasts { A B	1 3 } 4	5 0 } 5	2 0 } 2	3 1 } 4	1 1 } 2	1-5 } 0-3 } 2-5
Promegakaryocytes { A B C	4 9 16 } 29	8 12 10 } 30	5 12 4 } 21	5 18 11 } 34	6 6 14 } 26	4-8 } 6-18 } 21-34 4-16 }
Megakaryocytes { A B C D	23 10 33 0 } 66	12 25 24 0 } 61	11 25 35 0 } 71	11 26 24 0 } 61	10 12 37 3 } 72	11-23 } 10-26 } 61-72 24-37 } 0-3 }
Asynchronous Megakaryocytes	0	4	5	1	0	0-5
Active Megakaryocytes { B C D	22 49 0	37 34 0	37 39 0	45 35 0	19 51 3	19-45 } 34-51 } 0-3 }

TABLE 2.—*Differential Counts (in %) of Megakaryocytes in Idiopathic Thrombocytopenic Purpura (Group 1)*

	Case No					
	J <sup>1</sup> O	M <sup>2</sup> R.	G <sup>3</sup> S	R. <sup>4</sup> J	P <sup>5</sup> S	6 L. G. M. (In re- mission)
Platelets in cu mm	92,000 (D†)	33,480 (D)	47,190 (D)	—	34,380 (D)	221,970 (F‡)
Megakaryocytes	increased	increased	increased	increased	increased	normal
Amitotic forms	1	2	1	2	1*	0
Mitotic forms	1	0	0	2	1	0
Megakaryoblasts { A B	4 3 } 7	5 0 } 5	3 0 } 3	4 3 } 7	0 0 } 0	2 0 } 2
Promegakaryocytes { A B C	22 9 0 } 31	27 3 0 } 30	21 5 0 } 26	22 3 0 } 25	18 10 0 } 28	11 14 0 } 25
Megakaryocytes { A B C	46 10 0 } 56	50 5 0 } 55	58 8 0 } 66	53 8 0 } 61	50 17 0 } 67	23 36 9 } 68
Asynchronous Megakaryocytes	4	8	4	3	3	5
Active Megakaryocytes { B C	22 0	8 0	13 0	14 0	18 0	50 9

\* B cell

† D = wet smear method of Dameshek

‡ F = dry method of Fonio

TABLE 3.—Differential Counts (in %) of Megakaryocytes in Chronic Splenomegalies (Group II)

	Case No					
	7 A B	8 R O	9 R V	10 F M	11 S B	12 B A
Platelets per cu mm	112,000 (D)	173,040 (D)	131,760 (F)	102,000 (D)	66,690 (F)	108,000 (F)
Megakaryocytes	normal	normal	normal	normal	normal	normal
Amitotic forms	6	2	1	4	1	2
Mitotic forms	0	0	0	1*	1*	1
Megakaryoblasts	{ A B } 5 } 6	{ 9 } 9	{ 4 } 4	{ 7 } 7	{ 1 } 1	{ 6 } 7
Promegakaryocytes	{ A B C } 12 } 23 1 }	{ 17 } 20 3 } 0	{ 13 } 21 8 } 0	{ 11 } 23 12 } 0	{ 18 } 26 8 } 0	{ 15 } 24 9 } 0
Megakaryocytes	{ A B C } 34 } 63 25 } 4	{ 36 } 62 25 } 1	{ 28 } 71 32 } 11	{ 35 } 65 29 } 1	{ 32 } 68 36 } 0	{ 42 } 65 20 } 3
Asynchronous Megakaryocytes	2	7	3	0	3	1
Active Megakaryocytes	{ B C } 34 } 5	{ 28 } 1	{ 40 } 11	{ 42 } 1	{ 45 } 0	{ 30 } 3

\* B cell

TABLE 4.—Differential Counts (in %) of Megakaryocytes in Miscellaneous Diseases (Group III)

	Case No							
	13 P F	14 J V	15 C B	16 M P	17 S C	18 A P	19 M G	20 C R
Platelets per cu mm	52,400 (D)	—	39,200 (D)	372,900 (D)	62,200 (D)	149,240 (F)	—	35,952 (D)
Megakaryocytes	de creased	nor mal	de creased	de creased	de creased	nor mal	markedly decreased	de creased
Amitotic forms	2	3	1	2	0	0	6	0
Mitotic forms	0	1*	0	0	0	0	0	0
Megakaryoblasts	{ A B } 4 } 4	{ 5 } 6	{ 9 } 9	{ 0 } 0	{ 0 } 0	{ 1 } 1	{ 0 } 0	{ 0 } 0
Promegakaryocytes	{ A B C } 11 } 13 2 } 0	{ 10 } 16 6 } 0	{ 9 } 10 1 } 0	{ 12 } 18 6 } 0	{ 6 } 8 2 } 0	{ 2 } 6 2 } 2	{ 3 } 10 6 } 0	{ 6 } 6 0 } 0
Megakaryocytes	{ A B C } 56 } 77 21 } 0	{ 25 } 72 43 } 4	{ 50 } 60 10 } 0	{ 18 } 80 36 } 26	{ 66 } 80 14 } 0	{ 44 } 92 35 } 13	{ 58 } 79 17 } 3	{ 84 } 94 10 } 0
Asynchronous Megakaryocytes	4	2	20	0	12	1	3	0
Active Megakaryocytes	{ B C } 23 } 0	{ 51 } 4	{ 11 } 0	{ 42 } 26	{ 16 } 0	{ 37 } 15	{ 24 } 3	{ 10 } 0

\* B cell



TABLE 12 — Summary of Important Data in Cases of Idiopathic Thrombocytopenic Purpura (Group 1)

Case No	Clinical Findings	Hemoglobin Gm per 100 cc	Erythrocytes Millions	Reticulocytes %	Leukocytes Thous	Leukocyte Differential Count Percentage								Platelets Thousands	Bleeding Time	Clotting Time	Clot retraction	Bone Marrow	
						B	E	M	J	St	S	Ly	Mo	Plasma Cells	Normoblasts			General Impression	Myceloid erythroid ratio
1	Severe epistaxis purpura all over the body No history of previous bleeding Spleen and liver not palpable	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	markedly delayed	hyperplastic	1 6
2	Spontaneous bleeding from mouth and generalized purpura No history of previous bleeding Spleen and liver not palpable	5 61	86 5	15 7	0	0	0	0	0	29	52	11	5	2	1	33 5 (D)	normal	hyperplastic	3 4
3	Proluse epistaxis and ecchymoses in legs and arms No history of previous bleeding Spleen and liver not palpable	6 81	91 4	8 21 5	0	2	1	1	1	15	48	26	4	2	0	34 4 (D)	normal	hyperplastic	1 7
4	Bleeding of gums and generalized purpura No history of previous bleeding Spleen and liver slightly palpable	10 7 4	0 4 3	2 4 55	2	1	0	2	2	26	30	31	8	0	0	64 6 (D)	normal	hyperplastic	0 9
5*	Profuse bleeding from nose and gums No purpura History of frequent epistaxis and liver not palpable	8 3 2	62	—	6 55	0	0	0	2	14	32	38	6	8	0	117 9 (F)	normal	hyperplastic	6 5
6†	Periodic epistaxis and petechial hemorrhages in the skin for ten years Liver and spleen not palpable	13 6 4	53	—	8 0	0	2	0	0	2	65	28	3	0	0	221 9 (F)	normal	normo-cellular	3

\* Bone marrow was secured immediately before and peripheral blood immediately after fresh whole blood transfusion

† In partial remission

TABLE 3a—Summary of Important Data in Cases of Group II

Case No	Diagnosis	Hemoglobin Gm per 100 cc	Erythrocytes Millions	Reticulocytes %	Leukocytes Thousands	Leukocyte Differential Count Percentage										Platelets Thou sands	Bleeding Time	Clotting Time	Clot retraction	Bone marrow		Remarks
						B	E	M	J	St	S	Ly	Mo	Plasma cells	General Impression					Mye- loid eryth- roid ratio		
7	Malaria (P vivax)	9.34	0	1	3.55	2	7	0	1	20	32	30	6	1	112	0	normal	normal	hyper plastic	1/3	No hemorrhage	
8	Malaria (Unclassified)	7.82	48	2.64	95	0	5	0	0	19	52	24	0	0	173	0	normal	normal	hyper plastic	1/6	No hemorrhage	
9	Malaria (Unclassified)	—	—	—	—	—	—	—	—	—	—	—	—	—	131	8	normal	normal	hyper plastic	2/5	No hemorrhage	
10	Malaria (P Vivax)	8.03	4	2.76	75	2	1	0	1	33	42	20	1	0	102	0	normal	normal	hyper plastic	1/8	No hemorrhage	
11	Schistosomiasis	10.13	7	—	3.7	0	17	0	0	4	58	20	1	0	66	69	normal	normal	hyper plastic	2	Echymoses in left arm and left lower eyelid History of occasional bleed- ing of gums and epistaxis Cir- rhotic liver	
12	Banti's syndrome	—	—	—	—	—	—	—	—	—	—	—	—	—	108	0	normal	normal	hyper plastic	1	No hemorrhage	

## MEGAKARYOCYTES IN NORMAL AND ABNORMAL INDIVIDUALS

TAB

Summary of Important Data in Cases of Group III

LEUCOCYTES IN NORMAL AND ABNORMAL INDIVIDUALS

Case No.	Diagnosis	Hemoglobin Gm per 100 cc.	Erythrocytes millions	Reticulocytes %	Leukocytes Thousands	Leucocyte Differential Count Percentage										Clot retraction	Bleeding time	Clothing time	General impression	Myeloid ratio	Remarks
						B	E	M	J	St	S	Ly	Mo	Plasma cells	Platelets Thous						
															(D)						
13	Lobar pneumonia with pneumococcus endocarditis (post mortem)	6.6	2.62	3.6	25.35	0	0	0	0	22	72	3	0	52.4	prolonged	normal	normal	markedly delayed	hyperplastic	7.3	Profuse epistaxis melena petechias in conjunctiva tongue body Liver palpable Spleen not palpable
14	Rheumatic pericarditis with superimposed endocarditis chronic splenic infarct	8.0	3.89	—	11.0	0	1	0	0	5	66	26	0	5.28	prolonged	normal	normal	normal	hyperplastic	6	Petechias all over the body Spleen barely palpable
15	Malaria (P. Vivax) complicated with infection of unknown cause	5.6	2.45	1.1	1.5	0	2	0	2	21	44	15	0	39.0	normal	normal	slightly delayed	hyperplastic	1.7	No hemorrhage Spleen palpable	
16	Subacute glomerulonephritis and amebic colitis (post mortem)	4.3	1.65	2.4	7.3	2	3	0	0	1	77	12	3	372.9	prolonged	normal	normal	hyperplastic	9	No hemorrhage Spleen not palpable Total NPN—129 mg per 100 cc of blood	
17	Caseous tuberculosis of both kidneys toxic splenitis (post mortem)	11.4	4.06	0.6	4.55	0	0	4	43	49	4	0	0	62.2	prolonged	normal	slightly delayed	slightly hyperplastic	2	Petechias all over the body more numerous in lower extremities Spleen not palpable	
18	Chronic myelogenous leukemia	8.1	2.87	2.2	656.0	Includes 1.5% of myeloblasts										149	normal	normal	markedly hyperplastic	61	No hemorrhage Spleen and lymph glands not palpable
19	Acute myelogenous leukemia	8.5	2.76	—	59.25	Includes 18% myeloblasts										—	normal	normal	markedly hyperplastic	80	No hemorrhage Spleen liver palpable
20	Idiopathic aplastic anemia with normo-cellular bone marrow	3.0	0.64	4.4	2.65	0	0	0	0	12	30	55	3	35.95	normal	normal	normal	normal	0.68	History of petechias six weeks prior to admission. No hemorrhage during period of observation. Spleen atrophic	

our observation, a patient may manifest at different periods clinical signs of acuteness or chronicity with corresponding variations in the megakaryocytic picture

The condition of the megakaryocytes in this disease has been the subject of much controversy. Dameshek and Miller<sup>2</sup> reviewed the various opinions, since Frank<sup>5</sup> for the first time suspected a causal relationship between thrombocytopenia and disturbance of the megakaryocytes. The majority of investigators incriminate a disordered spleen as the offending organ in the depression of circulating platelets. This view is supported by the increase of thrombocytes after splenectomy.

However, there is a disagreement over the mode of action. One school holds that the spleen produces a substance, still to be identified, which inhibits the production of platelets from the megakaryocytes.<sup>3</sup> The other postulates a phagocytic aggressiveness of the splenic reticulo-endothelial cells, the megakaryocytes all the while remaining intact.<sup>6</sup>

Inhibition of the platelet-producing activity of the megakaryocytes would naturally result in some kind of alteration in the cells. On this account, the quantitative and qualitative changes undergone by them have been the object of intensive research. Deficient platelet-formation and increase of immature cells had long been observed. Limarzi and Schleicher,<sup>7</sup> in addition, described a toxic form, characterized by a pyknotic nucleus and hyaline cytoplasm. More recently, Dameshek and Miller<sup>2</sup> determined the percentage of megakaryocytes active in the formation of platelets and counted only 14.4 per cent of the total number in the acute form and 34 per cent or less in the chronic. They also noted in both forms an increase in the total number of cells and in the percentage of megakaryoblasts and a proportionate decrease of promegakaryocytes.

In our cases (table 2), we have noticed a similar increase in the total number of megakaryocytic cells. Furthermore, the amitotic forms, found only occasionally in the normal, were invariably present. The megakaryoblasts were more than normal, only in the majority of cases. The values of promegakaryocytes and megakaryocytes were within the normal range. This contrasts with the findings of other investigators. We do not consider the presence of cells with pyknotic nuclei and agranular, hyaline cytoplasm as a specific anomaly in idiopathic thrombocytopenic purpura. This cell, which is included in our division of asynchronic megakaryocytes, may also be found in the normal (table 1).

It has been further observed that when the condition of the patient was severe, the megakaryocytes, both mature and immature, containing more than ten platelets in the cytoplasm were characteristically absent. When remission of symptoms took place, a small number of cells with more than ten platelets in the cytoplasm appeared. The subdivision of each developmental stage of the megakaryocytes, from the functional standpoint, into A, B, C and D cells is based on this observation.

Thus, reappearance of C cells may be considered a good prognostic sign. It certainly is a much more reliable index than the platelet count or the bleeding time, especially when whole blood transfusion was already given, before any hematologic analysis could be made.

In summary, the following changes were found in idiopathic thrombocytopenic

purpura (1) an increase in the total number of megakaryocytes, (2) invariable presence of amitotic forms, (3) occasional increase of megakaryoblasts, and, what is most important of all, (4) absence of C cells in the severe, and marked decrease of the same in the milder forms. All this no doubt points to a rapid multiplication of cells and decided inhibition of their platelet-forming activity without interference with the process of maturation.

*Group II* (table 3a) is composed of diseases associated with chronic splenomegaly. Investigation of the megakaryocytes in this condition has not yet been extensively undertaken. Besides Dameshek and Miller,<sup>2</sup> who included cases of secondary splenomegaly for comparison in their study of idiopathic thrombocytopenic purpura, Cartwright *et al.*<sup>8</sup> made a similar research in cases of kala-azar. They found a marked slackening in the fragmentation of platelets from megakaryocytic cytoplasm.

An analysis of our data on the megakaryocytes (table 3) in this group, which includes 4 cases of malaria, 1 of schistosomiasis and another of Banti's syndrome, shows (1) normal number of megakaryocytic cells, (2) presence of amitotic forms, (3) normal or increased megakaryoblasts, (4) a normal percentage of promegakaryocytes and megakaryocytes, and (5) diminished or absent C cells.

The last finding to my mind constitutes a convincing evidence in favor of the restraining influence exerted by the spleen over the megakaryocytic platelet-producing activity. This theory receives additional support from our investigation of other cases of secondary splenic enlargement which are not included in this report, because thrombocytopenia was absent. In them, the C cells were invariably below the normal percentage. The platelet count was maintained at a normal level by a compensatory increase in the total number of cells.

Case 8 of this group was splenectomized and after four days the platelets rose from 173,000 (D) per cu. mm. to 1,090,000 per cu. mm. and the C cells from 1 per cent to 52 per cent. This result tallied with the findings of American investigators after splenectomy in idiopathic thrombocytopenic purpura.

*Group III* (table 4a) comprises various diseases clinically unalike which nevertheless exhibited similar megakaryocytic reactions. Five were infectious in nature, one chronic myelogenous leukemia, one acute myelogenous leukemia and the last aplastic anemia with normocellular bone marrow. The important changes in the megakaryocytes (table 4) were (1) normal or low total number, (2) diminished promegakaryocytes and (3) complete disappearance or low percentage of C cells. In contrast with the first two groups, here there was a definite hastening of maturation.

Few investigators have studied in detail the changes of the megakaryocytes in these diseases. The data gathered prove to be interesting, since they offer a new, additional explanation for the spontaneous hemorrhages occasionally occurring in lesions of this group. In infection or toxemia, purpura is commonly attributed to a toxic injury suffered by the capillary walls,<sup>9</sup> in bacterial endocarditis to the blocking of small vessels by emboli,<sup>10</sup> and in primary blood dyscrasias to a simple reduction of the number of megakaryocytes.<sup>11</sup> While the purpurogenic action of these factors may not be denied, still our observation indicated that another cause may come into play.

## GENERAL CONCLUSIONS

Even in the normal (table 1) it is obvious that there exists only a loose connection between maturation and function of the megakaryocytes. While in the granulocytic and erythroid series, function is discharged only after attainment of maturity, in the megakaryocytes platelet-formation may take place even at the earliest stage of development. However, the more mature the cells are, the greater is the number of platelets produced.

This dissociation of function from maturation is exaggerated in the abnormal marrow. Cases in Groups I and II showed a distinct arrest of function, while the process of maturation remained normal. The process of maturation in cases of Group III was accelerated with associated depression of platelet-production. To complete the series of disturbances actually observed, we may mention that in many cases of iron-deficiency anemia, the megakaryocytes undergo a rapid rate of maturation, without interference with formation and delivery of platelets to the peripheral blood. In our experience we have not yet encountered a single instance wherein maturation was delayed.

From these facts, we conclude that the principles responsible for maturation and for platelet-production are not the same.

Comparing the differential counts of the megakaryocytes as found in normal and thrombocytopenic individuals, the number of platelets in the peripheral blood appears to be maintained at the normal level of 400,000 to 800,000 (D) per cu mm by 34 to 51 per cent of C cells. A diminution of these cells, unless compensated by a very excessive proliferation, as seen in some cases of chronic myelogenous leukemia, is reflected in the peripheral blood by a low platelet count. Disappearance of C cells corresponds to a thrombocytopenia of less than 100,000 (D) per cu mm.

The platelet count bears no relation to the percentage of B cells.

Comparing the data in tables 2, 3 and 4, it would appear that idiopathic thrombocytopenic purpura does not present a specific megakaryocytic picture, except in the acute stage, where the total number of cells is markedly increased and the C cells are totally absent. The conjunction of these two findings is not seen in other conditions.

The association of thrombocytopenia in any disease with disturbance of the megakaryocytes points to the fact that in general the cause of the former acts through the latter. Thrombotic thrombocytopenic purpura<sup>11-12</sup> may be an exception to this rule. However, in the cases reported no detailed examination of the megakaryocytes, as is possible only in direct smears of the bone marrow, has been made. Chronic splenomegalies secondary to various unrelated lesions offer an additional and strong clinical proof in support of Dameshek's concept of hypersplenism. (We are not yet in a position to evaluate the sequestration-and-phagocytosis theory of Doan<sup>13</sup>.)

The spontaneous hemorrhages in infection and toxemia and in certain primary blood dyscrasias pose a question to which the answer is yet to be found. *Is the thrombocytopenia of this nature also mediated through the spleen?* The latter, as is known, is involved in infection and leukemia, though in a different way. However, the prob-

lem becomes more puzzling when a megakaryocytic picture similar to that found in the two former conditions is associated with an atrophic spleen as in Case 20

#### SUMMARY

The megakaryocytes in 5 normal and 20 thrombocytopenic individuals were studied. The stages of development undergone by the cells were delineated and a new classification, according to maturation and function, was made.

Using this classification, the megakaryocytic reactions in the 20 cases of thrombocytopenia were grouped into three distinct types. The first is characterized by a rapid multiplication of cells and a marked inhibition of platelet-formation without change in the process of maturation. The second is similar to the first with this exception that the total number of cells is not increased. The third reaction shows a normal or low total number of cells, with inhibited platelet-formation and accelerated maturation.

From the collected facts, the following conclusions are inferred: (1) The factors of maturation and of platelet-production affecting the megakaryocytes are different. (2) Certain diseases may disturb either of the two processes or both. (3) The number of platelets in the circulating blood is directly related to the percentage of megakaryocytic C cells in general. (4) Lesions affecting the spleen usually check the fragmentation of platelets from the megakaryocytic cytoplasm. (5) Infection and primary blood disorders may cause changes in the megakaryocytes but the explanation for this is still to be investigated.

#### ACKNOWLEDGMENTS

The writer owes a debt of gratitude to the members of the Eye, Ear, Nose and Throat Department of the University of Santo Tomas and to the medical staff of the North General Hospital for the use of some of their patients in this investigation.

#### REFERENCES

- <sup>1</sup> DE LA FUENTE V. Megakaryocytic reaction localized to the bone marrow. *Arch. Int. Med.* 71: 387-404, 1946.
- <sup>2</sup> DOWNEY H. The origin of blood platelets. *Folia haemat.* 15: 25, 1913.
- <sup>3</sup> DAMESHEK W. AND MILLER E. The megakaryocytes in idiopathic thrombocytopenic purpura: a form of hypersplenism. *Blood* 1: 27-51, 1946.
- <sup>4</sup> DOWNEY H., PALMER M., AND POWELL L. The origin of the megakaryocytes in the spleen and liver in a case of atypical myelosis. *Folia haemat.* 41: 55, 1930.
- <sup>5</sup> FRANK quoted by DAMESHEK AND MILLER.<sup>3</sup>
- <sup>6</sup> DOAN, C. A., AND WRIGHT C. Primary congenital and secondary acquired splenic panhematopenia. *Blood* 1: 10, 1946.
- <sup>7</sup> LIMARZI L. R., AND SCHLEICHER E. M. The reaction of peripheral blood and bone marrow in chronic hemorrhage and in essential thrombopenic purpura. *J. A. M. A.* 114: 12-18, 1940.
- <sup>8</sup> CARTWRIGHT, G. E., HUI LAN C. AND AN C. Studies on the pancytopenia of kala azar. *Blood* 3: 249-275, 1948.
- <sup>9</sup> BOYD W. *The Pathology of Internal Diseases*, ed. 4. Philadelphia: Lea & Febiger, 1944. P. 421.
- <sup>10</sup> DOCK, W. Bacterial endocarditis. In STROUD W. *Diagnosis and Treatment of Cardiovascular Disease*, ed. 3, vol. I. Philadelphia: F. A. Davis Co., 1945. P. 151.
- <sup>11</sup> SINGER K., BORNSTEIN F. R. AND WILE S. A. Thrombotic thrombocytopenic purpura. *Blood* 2: 542-554, 1947.
- <sup>12</sup> FITZGERALD P. J., AUERBACH O. AND FRAME E. Thrombotic thrombocytopenic purpura (Platelet thrombosis of capillaries, arterioles and venules). *Blood* 2: 519-541, 1947.
- <sup>13</sup> DOAN C. A. Pan marrow hematopoiesis (L. Casei Factor) and splenic hematopenia. Experimental and clinical studies. *Proc. Institute of Medicine of Chicago*, Vol. 16, No. 6, June 15, 1946.

## PLATELET THROMBOSIS IN HUMAN HEMOSTASIS

### A HISTOLOGIC STUDY OF SKIN WOUNDS IN NORMAL AND PURPURIC INDIVIDUALS

By HOWARD D. ZUCKER, M.D.

THE IMPORTANCE of blood platelets and platelet thrombosis in the hemostatic mechanism of rats has recently been demonstrated by M. B. Zucker.<sup>1</sup> In view of the frequency of species variation in anatomy and physiology, it became of interest to obtain evidence for or against a comparable role of platelets in spontaneous human hemostasis. The desirability of such a study was confirmed by a search of the medical literature, very few observations on the mechanism of hemostasis in man have been published since 1882 when Hayem<sup>2</sup> and Bizzozero<sup>3</sup> first reported on the form and function of mammalian blood platelets. This void is in striking contrast to the extremely large literature dealing with the hemorrhagic diseases, blood coagulation, and with blood and isolated blood elements. Much circumstantial evidence concerning the mechanism of hemostasis in man is available from these data.

The technical difficulties in microscopic observation of human vessels during the arrest of hemorrhage have not been overcome. Aplitz<sup>4</sup> and M. B. Zucker<sup>1</sup> have studied mesenteric vessels of small mammals at magnifications sufficient to permit identification of the formed blood elements, this method is impracticable in man. Nail-fold capillary studies, such as those of Macfarlane<sup>5</sup> and of Magnus,<sup>6</sup> are of limited value, since the skin thickness and the refractive similarities of unstained platelets and plasma prevent definitive identification of the wound and vessel contents. Histologic study of fresh, human puncture wounds through serial sections was chosen as a method which would permit analysis of some of the factors involved in human hemostasis. Shortly after these experiments were begun, it was discovered that Aplitz<sup>7</sup> had earlier applied serial section methods to bleeding time puncture wounds, obtained at autopsy.

#### METHODS

The skin of the neck or abdomen of anesthetized surgical patients was cleansed with alcohol and punctured by a small Hagedorn needle, each puncture was designed to penetrate 3-4 mm. beneath the surface. Many puncture wounds in control and purpuric patients failed to exhibit macroscopic bleeding and in such instances puncture was usually repeated at a distance. Microscopic hemorrhage was invariably found when such grossly dry puncture wounds were studied. When visible bleeding ensued, the local bleeding time was recorded by absorption of the drops of blood at half minute intervals.<sup>8</sup>

After puncture, the patient was prepared for operation in the usual manner except that scrubbing directly over the wound was avoided. At the time of his initial incision usually fifteen to twenty minutes after the test, the surgeon removed a small ellipse of skin and subcutaneous fat containing the puncture. The biopsy specimen was trimmed to appropriate size with a straight razor, the epithelial surface flattened on blotting paper and the specimen fixed in half strength Zenker formol solution. After three and a

From the Laboratories, Division of Pathology, the Mount Sinai Hospital, New York City.  
Presented at the Forty-Fifth Annual Meeting of the American Association of Pathologists and Bacteriologists, March 13, 1948.



TABLE I

Case	Laboratory Data										
	Hb % RBC	WBC	Smear	Platelets	Bleeding Time	Clotting Time	Retraction	Pro- throm- bin	Tourni- quet Test	Marrow	Post Op
I S H	3 33 70%	11,800	Normal differential	20,000	Over 20 min	9 min	None in 24 Hrs	—	Posi- tive	Cellular No plate let formation	Slow return of plates and bleeding time Marrow normal in 1 wk plates seen
II M. L.	4 3 80%	7,000	Normal differential	Varied 10,000 to 80 000	22 min	11 min	None in 24 Hrs	100%	Nega- tive	Increased megakar yocytes in cellular marrow No plate formation	Platelets 20,000 on 3rd day 300 000 on 11th day
III A H	2 93 30%	10,550	Slight lymphocy- tosis absent	Under 10 000	Over 45 min	7 5 min	Begins 2 Hrs In comple at 16	85%	Posi- tive	Maturation arrest of megakaryocytes No plate forma- tion	1st 3 weeks Pl < 40 000, Bleeding time 13 min After 3 months Plates 80,000 RBC 46 M
IV S. C.	4 5 75%	10,000	Normal differen- tial Rare giant platelets	10 000	26 min	15 min	None	Nor- mal	Posi- tive	Increased megakar yocytes with no plate formation	Preop Microscopic he- matoma Postop No follow up

half hours the block was removed thoroughly washed in running water dehydrated, and paraffin-embedded in routine manner. Serial sections, at 7 micra, were cut through the entire block, and mounted in strips of 4 to 7 sections per slide. Invariably a few sections were lost, but not many from any series. Most slides were stained in Mallory's phosphotungstic acid hematoxylin solution which permits differentiation between erythrocytes, fibrin and platelets and platelet products by structural and staining qualities. Occasional slides were stained with hematoxylin and eosin, or with Weigert's elastica.

Blocks of skin from three patients with normal bleeding times were studied. Two were from the necks of hyperthyroid patients and one from the abdomen of a patient with chronic peptic ulcer. All were removed under ethylene-ether anesthesia with scopolamine and morphine premedication. The local bleeding times were 1½ (no macroscopic bleeding), and 1½ minutes respectively. Further controls seemed unnecessary since in all three the essential findings resembled those reported in Aptiz's<sup>7</sup> autopsy controls.

Four blocks of abdominal skin removed from patients undergoing splenectomy for idiopathic thrombocytopenic purpura were studied. Abstracts of the clinical and pathologic data follow, the hematologic data are presented in table 1. Bleeding times of the experimental punctures are given with the histologic descriptions (Results).

### CASE REPORTS

I. S. H. (562354) A 26 month old male infant with negative family history, past history of recurrent eczema, had a history of intermittent red stools and gingival bleeding since birth. He had had two attacks of generalized purpura. Physical examination revealed closed fontanelles, liver one finger below the costal margin, many petechial and larger hemorrhages in the mouth and over the skin. Splenectomy was performed under ethyl chloride-ether anesthesia with atropine morphine premedication. The spleen weighed 48 Gm. (normal 33) microscopically it showed hyperplasia with slight, acute inflammatory changes, and was considered compatible with the diagnosis of thrombocytopenic purpura hemorrhagica. Postoperatively the patient had pyoderma which cleared under penicillin. Seven months after splenectomy he had no signs or symptoms of further purpura.

II. M. L. (566567) A 22 year old housewife gravid 1 para 1 with family history of allergies, had, herself had childhood eczema and frequent rashes, sometimes with pruritus and urticaria. For three years she had had a butterfly rash over the bridge of her nose which, diagnosed as discoid lupus, was treated with short courses of bismuth and one year before admission with three doses of mapharsen following which patient had three days of fever associated with leukopenia. During the year prior to admission the face lesions had become fainter. During that same year there was onset of a progressively severe hemorrhagic diathesis whose manifestations included severe menorrhagia, and showers of skin petechiae. Physical examination B. P. 106/66. Scaling erythematous lesions over nose. Generalized mucosal and skin petechiae and ecchymoses up to 4 cm. in diameter. Splenectomy was performed under ethylene-ether anesthesia with morphine and hyoscine premedication. The spleen weighed 160 Gm. showed no significant histologic changes and was considered compatible with a diagnosis of thrombocytopenic purpura. Postoperatively there was a three week febrile course attributed to a subphrenic hematoma, thereafter, the patient was well. Her husband, a physician reports her free of signs and symptoms of purpura 8 months after operation.

III. A. H. (565035) A 26 year old woman with negative family history and negative past history was admitted because of easy bruising and recurrent gingival bleeding during the preceding year. More recently epistaxes, prolonged menses and finally, tarry stools and smoky urine had been noted. Physical examination B. P. 110/60. Liver at costal margin. Generalized pinhead and larger mucosal and skin hemorrhages and ecchymoses. Splenectomy was performed under ethylene-ether-curare anesthesia with morphine and atropine premedication. The spleen weighed 86 Gm., and microscopically showed conspicuous blood cell formation mainly erythroblasts and megakaryocytes, a feature found occasionally in thrombocytopenic purpura with secondary anemia. For two and one half weeks the postoperative course was febrile, gingival bleeding continued and petechiae appeared over the lower extremities on ambulation. By two months the patient was sign and symptom free and she remained so until her most recent follow-up visit five months postsplenectomy.

IV. S. C. (565567) A 58 year old businessman with negative family history and without previous illness gave a six year history of gingival bleeding. For one year symptoms had been increasing with

easy bruising and one episode of tarry stools and smoky urine. Physical examination B P 176/80. Negative except for small ecchymoses on the right thigh and evidence of recent gingival hemorrhage. Splenectomy was performed under ethylene-ether anesthesia with morphine and atropine premedication. The spleen weighed 150 Gm and showed no significant histologic changes other than a slightly increased number of eosinophile and polymorphonuclear neutrophile leukocytes compatible with a diagnosis of thrombocytopenic purpura. The postoperative course was uneventful.

## RESULTS

*General Observations* In all of the blocks, the anatomy of the cutaneous blood vessels conforms in every essential to the description by Spalteholz.<sup>9</sup> Without his contrast injection technic, and without the physiologic methods of Chambers and Zweifach,<sup>10</sup> distinction between precapillary arterioles, thoroughfare channels, and postcapillary venules is uncertain, since all are of the order of 8 to 14 micra in diameter, and since their muscular components are attenuated. Identification of these small vessels by tracing them through the series to easily identifiable arterial and venous radicals of the next largest order, is both tedious and difficult, since these larger arterioles and venules characteristically run in close association. No attempt has been made to distinguish between the majority of these minute vessels since the sequels of hemorrhage are found to be the same in all.

Unusual valves, briefly mentioned by Spalteholz,<sup>9</sup> are seen in several blocks. These valves lie in the mouths of small tributary venules exactly at their entrance into a large venule of the 4th venous (subcutaneous) network, the anatomy of these valves is clearly established by observation through the series. Only a few tributary venules exhibit such valves, and conventionally placed valves are also seen in the large venules.

In contrast with the findings of Spalteholz, who reports single capillaries, pre- or postcapillary vessels are often seen dividing into two true capillaries near the peak of a papilla. This contrast may be due to local variation determined by choice of different skin areas.

The structure of the experimental wounds is variable, despite attempts to puncture the skin with uniform technic. None of the wound mouths gape widely. Most of the wounds have a characteristic, narrow, V shape, but while some penetrate only half of the skin thickness, others extend into the subcutaneous fat. Most striking is the variation in the number and caliber of the vessels which have been cut. In blocks where few vessels have been cut, sizeable vessels which have escaped puncture can often be traced through the series within a few micra of the wound edge.

No difference can be made out between the endothelium of blood vessels of control and of purpuric patients, or between the endothelium of smaller and larger vessels. In normal and purpuric skins, the endothelium of opened vessels usually exhibits two or three flattened, pyknotic nuclei near the lips of the vascular wound, the remaining endothelial nuclei are normal.

Although segments of true capillaries are recognized with moderate ease throughout the sections, only rare capillaries can be identified that have been opened by the punctures. Presumably recognition of these vessels is hindered by

the traumatic distortion of their endothelium, and, possibly, by collapse and by endothelial agglutination

*Normal Skin* All of the needle puncture wounds in normal skin are filled by masses of well preserved erythrocytes and strands of fibrin. The fibrin appears in strands of varying density, or in the form of fine needles. The heaviest fibrin bands are oriented in the long axis of the wound, roughly perpendicular to the skin surface, less dense strands are seen threaded in all directions between the red cells. The wound margins have an incomplete, thin fibrin cover, where this is lacking the tissue edges are usually bare, but, in places, platelet masses (*viz* infra) form the lining. Occasional white blood cells, single or in groups, are seen in the coagulum. Smaller and larger refractile fragments of the horny layer may be found in the wounds, some in deep or superficial recesses, others lying at the side of the red cell-fibrin clot, none have tamponaded blood vessels in the manner described by Apitz.<sup>7</sup> No inclusions of other epidermal layers are seen in the wound depths, although small, doorlike strips of the entire epidermis, with distorted, pyknotic nuclei, may be seen opening either inwards or outwards at the wound mouths.

The difficulty of identifying opened true capillaries has been described. No capillaries are plugged by platelet masses. Such open capillaries as are identified have fibrin strands sealing their exposed lips, the sealing fibrin strands lie along the wound margin, and do not enter the lumens of the transected vessels.

One or more precapillary arterioles or postcapillary venules have been transected by each puncture. In these normal skins, every such vessel, save two, is sealed by a small or large platelet plug. A number of loose blood platelets are seen in the stumps of some of these transected vessels, giving the illusion of streaming towards the wound. The stumps are sealed, however, by a densely packed platelet mass, 90 per cent of which protrudes from the stump into the needle puncture tract. Occasional well preserved platelets are made out within the masses, but the bulk of the platelets are already involved in the process of fusion (viscous metamorphosis) which is characteristic of these elements in animal experiments<sup>2, 3, etc</sup> and *in vitro*.<sup>2, 11, etc</sup> These coarsely granular masses, which stain blue-gray in phosphotungstic acid-hematoxylin, bear no resemblance to nearby red cells. At the margins of the fused platelet thrombi there is, characteristically, a thin, darkly stained band which resembles fibrin, but which does not have the clear definition of the fibrin in the adjoining clot. Similar condensed bands, never more than one or two, may sometimes be seen within the plug, but discrete fibers or needles of fibrin are never seen. Single, well preserved red or white blood cells occasionally lie within the platelet thrombi. Exceptions to this mechanism of platelet thrombosis are seen in pre- or postcapillary vessels in two different blocks. In one of these a red cell can be seen squeezing out of an unclosed vessel into the puncture tract, in the second instance a similar vessel, well beneath the germinal layer, is tightly covered by a portion of the fibrin lamella lining the wound tract. Fibrin is not seen within the lumens of any of these thrombosed vessels.

In many sections platelet masses can be seen lying within the wound tract without apparent relation to an opened vessel (fig. 1). Invariably these masses

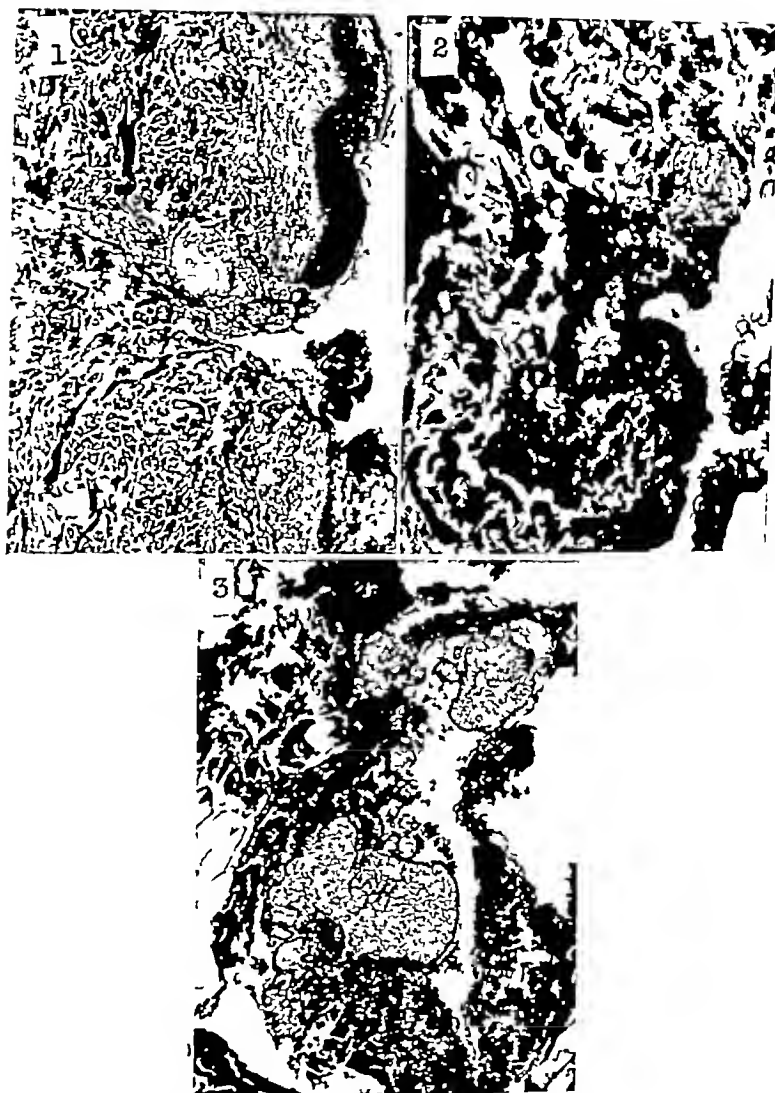


FIG. 1 —PUNCTURE WOUND OF NORMAL SKIN. A fused platelet mass is seen lying in the red cell fibrin coagulum. The vessel from which the mass originates is not seen in this section.

FIG. 2.—55 MICRON ARTERIOLE IN NORMAL SKIN. Extravasated erythrocytes lie in the perivascular space. The vascular defect is filled by a platelet thrombus which is divided by a remaining shred of vessel wall. Within the thrombus many individual platelets can be identified as well as two trapped leukocytes and several erythrocytes.

FIG. 3 —250 MICRON VENULE IN NORMAL SKIN. A large platelet thrombus seals the gaps in the injured vein wall and obstructs some of the vessel lumen. The thrombus exhibits prominent marginal lamination. India Ink discolors the adjacent fat.

can be traced, through the series, to the stump of a severed small vessel. Thus, in three dimensions, one may picture the normal puncture wound as a tubular tract into which a varying number of small vessels open. Each opened end is sealed by a platelet cork, the bulk of which projects into the tube. The remainder of the tube is filled with a red cell-fibrin clot reaching to, but not beyond, the skin surface, infrequent white cells and small, horny-layer foreign bodies are scattered through the clot.

No vessels greater than 250 micra in diameter have been injured by the experimental punctures. In one block of normal skin an arteriole, measuring 55 micra in its smallest diameter, has been perforated. This vessel lies at the surface of the subcutaneous fat, near the margin of the block. Since no continuity can be demonstrated between this injury and the needle wound, it is believed that the arteriolar damage was sustained during the process of excision. The arteriole is surrounded, along most of its course in the block, by a collar of extravasated erythrocytes which infiltrate into the adjacent fat. Through many sections of the series a defect can be traced in the vessel wall, more than one-third of the circumference is involved at the site of maximum damage. The margins of this vascular wound are not entirely smooth, and a remaining shred of wall, at one end of the series, gives the illusion of two adjacent arterial wounds rather than of a single one. The defect in the arterial wall is completely filled by a fresh platelet thrombus, composed, in large areas, of tightly packed blood platelets so well preserved that each may be distinctly made out. In other areas fusion has occurred and only occasional individual platelets can be made out, here the structure resembles the plugs described in the smaller vessels. Occasional red or white blood cells are included in the thrombus which contains no fibrin, but exhibits the marginal band described in the smaller plugs. The platelet thrombus lies almost entirely within the gap in the arterial wall, some knobby excrescences protrude into the adjacent fat, or, without appreciable obstruction, into the arterial lumen (fig. 2).

A 250 micron venule of the 4th network has been opened in a different case. Again the defect is unrelated to the experimental puncture. This was the only experiment in which an attempt was made to mark the region with an intracutaneous india ink dot. The sections show that the tattooing needle penetrated subcutaneously, and destroyed a considerable segment of vein wall. As with the artery, the vein is surrounded by a pool of extravasated blood, and the defect in its thin wall is entirely sealed by a platelet plug. This is a massive thrombus of somewhat looser construction than the others, for the individual platelets are clearly seen with hairlike, stellate processes radiating between them. In some sections more than half of the circumference of the vein wall is missing, and has been replaced by the thrombus, the vascular lumen is two thirds obliterated at some levels (fig. 3). Other, smaller venules show an entirely similar picture on a reduced scale, one exhibits streaming of individual platelets toward the platelet plugged opening.

No unsealed, injured arteries or veins are found in these normal blocks, except at the extreme periphery where microtome and fixation artefacts are too frequent to permit analysis in serial section.

In uninjured larger vessels, in interstices in the subcutaneous fat, and within

some of the wounds one may see masses of finely granular and linear material which usually stains blue with phosphotungstic acid-hematoxylin, but occasionally stains buff. The granules never exceed 0.4 micra in diameter, and the largest lines are not more than 0.4 by 1 micra, although they tend to coalesce. This material is seen in all my blocks (normal and purpura). It corresponds in appearance to the illustration in Fitzgerald's recent paper<sup>1</sup> on acute febrile thrombocytopenic anemia (his fig. 1). In this laboratory identical material has been seen in the portal vein radicles of routine autopsy liver sections in a variety of conditions, particularly in cases with conspicuous hepatic edema. No systematic search of other organs has been made. This material consists, therefore, of particles much smaller than blood platelets, does not resemble the products of platelet fusion as described here and elsewhere, is seen in conditions unassociated with platelet thrombosis, and is presumed to have no physiological bearing on the present data. I believe it to be a granular precipitate of plasma protein.

*Skin in Idiopathic Thrombocytopenic Purpura.* Case I had two punctures one of which failed to bleed macroscopically, the second puncture bled for 15 minutes, until excision. The sections exhibit a penetrating, superficial wound which, running at an angle of 30° to the skin surface, can be followed through much of the series. This extensive wound is filled with uncoagulated blood, and I believe that it represents enlargement of the second experimental puncture consequent to 15 minutes of active bleeding. A few threads of fibrin are scattered among the red cells, but no platelets are found in any section. Various severed or punctured vessels are encountered. One is a capillary from which red cells can be seen escaping into the wound. A number are opened pre- or postcapillary vessels, none of these are sealed, and most contain and are surrounded by red cells. Several are unsealed, transected or punctured venules (fig. 4). Another is a moderate sized arteriole which opens into the wound from below. No large, injured vessels are encountered.

A second, very superficial puncture wound is seen at one end of this block. Although the puncture never bled macroscopically, it is filled with a red cell fibrin coagulum whose denser fibrin strands are oriented perpendicular to the skin surface. No opened vessels of any caliber are identified in the series, and no platelets or platelet masses are present.

*Case II.* This puncture wound bled for three minutes and was removed at fifteen minutes. It is seen to penetrate to the deepest layers of the corium, but not into the fat. The wound contours are entirely similar to the controls, and the tubular tract is filled with a red cell-fibrin clot. In the depths the densest fibrin strands are oriented in the wound axis, perpendicular to the skin surface, but near the wound mouth the dense strands form a transverse dam, paralleling the skin surface (fig. 5). The few vessels found opening into the tract are all sealed by fibrin strands, none are greater than 10 micra in diameter. No blood platelets or platelet masses are seen. There is prominent dilatation of many capillaries and small vessels.

*Case III.* Two punctures were made, each of which bled for five minutes. In an effort to obtain material of greater interest, a third puncture was made with a



FIG 4—MODERATE SIZED VENULE IN THROMBOCYTOPENIC PURPURA. Blood is seen escaping into the perivascular tissues from a small perforation. Platelets and fibrin are absent.

FIG 5—PUNCTURE WOUND IN THROMBOCYTOPENIC PURPURA. No platelet masses are present. Several dense superficial strands of fibrin parallel the skin surface.

FIG 6—SMALL MUSCULAR VESSEL (12 MICRA) IN PURPURA. (a) Two vessels approach the wound edge which is coated by thin fibrin strands and enmeshed red cells. The opening between the vessels is an artefact. (b) The lower vessel is open (→) its lips are sealed by the overlying fibrin-red cell clot. The artefact is still seen.



large, cutting edged, surgical needle, this wound bled freely for ten minutes, until the entire block was removed

Both of the smaller wounds are filled with fibrin- red cell clots. One, near the edge of the block, has been so distorted by fixation and cutting that it cannot be followed in series. The other is similar in contents, and in the size and appearance of its cut vessels, to the tract of Case II. Unlike Case II the denser fibrin strands in this puncture are oriented perpendicular to the skin surface.

The third wound is U shaped, and about two and one-half times greater in diameter than any of the other wounds studied. It penetrates to the base of the corium. The superficial portions of the wound are empty, perhaps due to falling out of their contents. At the wound margins there is a thin fibrin network with enmeshed red blood cells. The deeper portions of the wound contain a red cell fibrin coagulum in which the uppermost, dense fibrin strands parallel the skin surface. Some opened pre- and postcapillary vessels are encountered at the wound edges, most of these are sealed by fibrin (fig. 6), but some lack seals, as do several capillaries. No larger vessels have been cut. No platelets are seen. Small, finely granular masses suggesting plasma precipitate are present.

*Case IV* Two punctures were made, both of which oozed briefly, the local bleeding times were less than  $\frac{1}{2}$  minute. The block was excised at 20 minutes. Both wounds have the characteristic, narrow V shape, and are filled with fibrin red cell coagula. One penetrates to the deepest corium, ending among some sweat glands, the other penetrates only half of the skin thickness. The denser fibrin strands in both wounds are oriented in the wound axis, perpendicular to the skin surface. A few cut pre- and postcapillary vessels are identified in each wound, very few in the smaller. These vessels, as well as the rare capillaries identified at the wound edges, are sealed by fibrin. No platelets or platelet masses are seen.

### DISCUSSION

*Platelet Agglutination* Intravascular thrombosis, rather than hemostasis, was the subject of Bizzozero's<sup>3</sup> experiments. His investigations, confirmed and elaborated by others,<sup>12-18</sup> have clearly established that mammalian blood platelets rapidly agglutinate at the site of vascular injury. Here they form intravascular platelet thrombi, with the inclusion of occasional red or white blood cells. The massed platelets quickly undergo the same viscous metamorphosis that is seen *in vitro*,<sup>11</sup> and they subsequently become surrounded by secondary clot composed principally of red cells and fibrin. Welch<sup>16</sup> and, particularly, Aschoff<sup>18</sup> have offered much evidence for the identity of experimental thrombi with those encountered in human pathology.

Hemostasis effected by platelet agglutination was first demonstrated by Hayem<sup>2</sup> in an experimental wound of a dog's jugular vein. Lubnitzky,<sup>19</sup> in 1885, reported hemostatic platelet thrombosis in similar wounds of rabbits' cranial arteries. After a lapse of forty years, this early work was confirmed by Apitz<sup>4</sup> and M. B. Zucker,<sup>1</sup> and extended by them in studies involving experimental interference with the hemostatic mechanism, such as the use of heparin, dicumarol, or antiplatelet serum. The present observations confirm Apitz's demonstration that blood platelets also

agglutinate in the defects of cut skin vessels in man. Such platelet thrombi have been seen in vessels ranging from 8 to 250 micra in diameter.

The rate of platelet agglutination in vascular wounds was first studied by Lubnitzky.<sup>19</sup> Her histologic studies show that, in rabbits, an incomplete thrombus fills the arterial gap within fifteen seconds, but that open pathways remaining in the platelet plug are not closed until the end of the first minute. The modern workers<sup>1-4</sup> have watched the formation of platelet thrombi in vascular wounds of small animals, these thrombi, which appear within ten to thirty seconds, become hemostatically effective within four minutes, despite gentle irrigation. My observations indicate that the rate of platelet agglutination in man approximates that seen in other mammals. Thus, viscous metamorphosis seen in blocks removed as early as ten minutes after experimental puncture, indicates that the platelet thrombi have been present for some time. In one instance, an arteriolar wound, thought to have been incurred during the removal of the block, is plugged by a mass of morphologically distinct, unfused platelets, presumably this plug formed in thirty seconds or less.

The normal adhesiveness and cohesiveness of platelet agglutinates has not been quantitatively studied. Lubnitzky<sup>19</sup> showed that unsupported platelets can not resist the blood pressure of large arteries, since platelet thrombosis could be produced in rabbit arteries only if moderate proximal pressure were applied. The present experiments show that in man, even without fibrin backing, a platelet thrombus can resist the effective blood pressure in an arteriole of 55 micra.

The structure of platelet thrombi varies somewhat, according to the nature of the vessel thrombosed. In larger arterioles the platelet masses lie chiefly within the mural defect, but exhibit small intraluminal projections, larger projections may be prevented by the breaking off of tiny emboli, a phenomenon described in experimental animals.<sup>1-4</sup> The platelet thrombi in some venules exhibit large intraluminal projections, probably because fragmentation is less frequent with lower blood pressure. In smaller vessels the plugs are more like mushrooms, with a small stalk lying in the vascular defect, and a larger, extravascular projection. Neither Apitz<sup>7</sup> nor I have seen platelet thrombi in true capillaries.

The factors underlying the deposition and agglutination of platelets are not clarified by my data. The exhibition, at the lips of each severed vessel in the sections, of one or two distorted endothelial nuclei, is no reason to assume a causal relationship with the arrest of platelets at these sites. And, although this endothelial alteration is present in every vessel, no platelets have accumulated at the mouths of opened capillaries, and none have accumulated in the stumps of the cut vessels in cases of thrombocytopenic purpura. The present histologic studies permit no interpretation of the possible hydrodynamic factors<sup>20</sup> involved in platelet agglutination, nor of the chemical or physical factors which render blood platelets agglutinable.<sup>21</sup>

*Fibrin Deposition.* In man, as in experimental animals,<sup>4</sup> there is no evidence that fibrin plays a significant role in platelet thrombus formation. The plugs described by Apitz<sup>7</sup> and by myself contain occasional dense, linear strands, and are usually outlined by thin, perimetric bands, we both consider this material to be

fibrin, but do not believe that it is essential in the structure of platelet thrombi. It is possible that minute quantities of fibrin, not identifiable by routine histologic methods, are formed at the platelet interfaces during thrombosis.

The present observations support those of Schimmelbusch,<sup>11</sup> who first opposed the theory<sup>3</sup> that platelets are the anatomic nidus from which fibrin strands arise. Virtually no fibrin is seen in the pools of extravasated blood which are found surrounding large subcutaneous arterioles and venules exhibiting platelet thrombi. Conversely, abundant fibrin is seen in the platelet free puncture wounds of purpuric patients. Except for the thin perimetric lamellae, the orientation of fibrin within the normal tracts seems unrelated to distribution of the platelet thrombi.

*Hemostasis.* Any tenable theory of normal, spontaneous, human hemostasis must account for the arrest of bleeding within a stated time interval (one to three minutes for small skin wounds<sup>22</sup>), and must account for the prolonged absence of renewed bleeding. Doubtless many factors are operative in the hemostatic mechanism, as is clearly brought out in Tocantins' recent review.<sup>23</sup> Whatever the contributory factors may be, bleeding stops when no more red cells escape from opened vessels, and renewed bleeding can only be prevented by the permanent sealing of opened or severed vessels. An essential problem in hemostasis is, therefore, the nature of the hemostatic vascular seal.

Fibrin formation, i. e., coagulation of the blood, has often been suggested as the mechanism whereby opened vessels are sealed. Hayem<sup>2</sup> raised the objection that fresh blood is continually passing between the lips of vascular wounds, and that such blood will not clot, *in vitro*, within many minutes. His objection seems valid today. In the laboratory, even with excess thromboplastin, human blood does not clot in less than eleven seconds<sup>4</sup>, therefore, even if fibrin be a sufficient seal, a given drop of blood must be delayed for eleven seconds at the lip of the severed vessel if hemostasis is to occur.

Macfarlane,<sup>5</sup> in his stimulating and widely quoted review, has suggested that vascular contraction may account for hemostasis by providing sufficient time for coagulation of extravasated blood. As a corollary it is postulated that fibrin is an adequate hemostatic agent. Common surgical experience contravenes the assumption that red clot is likely to act as an adequate seal for larger vessels. Unligated vessels, in the tonsillar fossa for instance, frequently recommence bleeding despite the presence of abundant red clot. Although Tannenberg and Herrman<sup>24</sup> offered experimental evidence that contraction of small vessels may prevent blood loss, their experiments do not establish that such vasoconstriction is followed by the permanent arrest of hemorrhage. It is possible that in smaller muscular vessels, under some circumstances (*viz. infra*), fibrin may be an adequate seal. Chambers and Zweifach,<sup>10</sup> and the Clarks,<sup>6</sup> have presented evidence that contraction of true capillaries does not take place. Consequently, in the absence of definitive visualization or of histologic confirmation, it is difficult to accept hemostasis by nail-fold capillary contraction, as reported by Macfarlane<sup>5</sup> and by Magnus.<sup>8</sup>

Hayem<sup>2</sup> stated that blood platelets are the essential factor in normal hemostasis, and that other elements are merely accessory and secondary. His theory conforms to modern evidence. In man, Apitz<sup>7</sup> and I have shown that platelet thrombi

normally form in most cut skin vessels larger than capillaries. The present experiments also show that human platelet thrombi can form within the expected time span, and that such thrombi are pressure resistant. The importance of platelets in the normal mechanism preceding clotting is further indicated both by the failure of fibrin formation in briskly bleeding thrombocytopenics who had normal clotting and prothrombin times, and by the delay of fibrin formation in other thrombocytopenics.

One might then picture platelet thrombi as coffer dams which, aided by local vasoconstrictor mechanisms, stop or slow the blood flow to the point where extravasated blood within the wound is given sufficient time to clot. Not all small vessels need have such a plug, for, once the flow has been considerably slowed coagulation will occur, and will seal any small vessels which have not yet accumulated sufficient platelets, rare fibrin-sealed small vessels have actually been seen in my normal blocks, none larger than 12 micra. The formation of fibrin within the wound tract, and its later retraction, represent the construction of a permanent, concrete dam which anchors and reinforces the older platelet thrombus coffers. Once the fibrin has buttressed the platelet thrombi, resumption of normal pressure relationships, as the vasoconstriction relaxes, is less likely to cause renewed bleeding. In contrast, coagulation alone, secondary to tight vasospasm or to obliteration of a vessel lumen by pressure, may fail to maintain hemostasis once the blood pressure is restored, for, without blood flow, thrombi can not have formed.

In capillary hemostasis the platelets may play no role. Neither Apitz<sup>7</sup> nor I have seen platelet plugs in human capillaries. Presumably the chief hemostatic factor in these vessels is the small difference between intracapillary and tissue pressures, this small pressure difference, and reflex contraction at the capillary mouths,<sup>10</sup> result in an ooze of blood from the opened capillaries which is sufficiently slow to permit coagulation within the wound. Evidence for this hypothesis is seen in the normal bleeding times of the puncture wounds of some thrombocytopenics, these wounds contain fibrin-red cell coagula sealing the capillaries, and also the few precapillaries which were cut. Apitz<sup>4</sup> has repeatedly seen immediate, permanent hemostasis in transected animal capillaries, due to endothelial agglutination, presumably this mechanism also occurs in man, and may account for the scant number of opened capillaries visualized.

The architecture of the fibrin meshwork within wounds has not been described in the literature. Consequently, in view of the small series reported here, the fibrin structure can not be stressed. The alignment of the denser fibrin strands in normal wounds may be in the direction of blood flow, for they are aligned in the long axis of the wounds. In contrast, the superficial fibrin layers, in two thrombocytopenics, bridge the wounds parallel to the skin surface, this configuration may be a concomitant of pathologic hemostasis.

The present results, and those of Apitz,<sup>7</sup> confirm the widespread, but previously unsupported, belief that there is great similarity between the human hemostatic mechanism and that of other mammals. The close parallel to the experimental observations of Apitz<sup>4</sup> and M. B. Zucker<sup>1</sup> suggests that associated physiologic

mechanisms, demonstrated in the laboratory but inaccessible to histologic study, also come into play in human hemostasis

**Bleeding Time** The clinical usefulness of the bleeding time is well established<sup>22</sup> A prolonged bleeding time is of pathologic and diagnostic significance, Quick<sup>24</sup> states that it is seen in the thrombocytopenic purpuras and in pseudohemophilia (thrombasthenia) The bleeding time may be normal in toxic purpuras, hypoprotrombinemia, hemophilia, and afibrinogenemia or hypofibrinogenemia

Apitz's studies of bleeding time tracts obtained at postmortem, and the present study of human puncture wounds\* obtained at biopsy give, for the first time, an accurate anatomical picture of such tracts The variability of depth of puncture, and the extreme variation in the size and number of vessels severed or punctured is an outstanding feature As commonly performed, some bleeding time punctures test only capillary bleeding time, while others test the entire mechanism of hemostasis These observations account for the common observation that repeated punctures are needed when hemorrhagic disease is suspected<sup>2</sup>, they probably explain the tendency for bleeding in thrombocytopenic purpura to vary in duration at different skin sites<sup>5</sup> Thus, in four blocks from thrombocytopenic patients, the bleeding time was normal when only capillaries were cut, but was prolonged when larger vessels were cut Clinically every effort should be made to produce deep, sufficiently wide cuts with a sharp blade, probably the size of the first few blots of blood, as suggested by Duke,<sup>8</sup> is the best index of an adequate bleeding time puncture

The prolonged bleeding time of idiopathic thrombocytopenic purpura is accompanied by the absence of platelet thrombi in the severed muscular vessels This absence of platelet plugs confirms Apitz's observations,<sup>7</sup> and is in agreement with the results obtained by Muller,<sup>27</sup> Apitz<sup>28</sup> and M. B. Zucker<sup>1</sup> in experimental thrombocytopenia Thus, loss of the primary hemostatic mechanism of platelet thrombosis, due to quantitative and, possibly, to qualitative platelet deficiency, seems to account for the abnormal bleeding of thrombocytopenic purpura Implication of other factors seems unnecessary If syneresis is of clinical significance, the mechanism substituted in thrombocytopenic purpura, namely coagulation, is also impaired

One might speculate that the prolonged bleeding in pseudohemophilia results from failure of platelet thrombosis on a qualitative basis, and that the notorious tendency towards renewed hemorrhage in hemophilia<sup>24</sup> is based on the lack of a strong fibrin backing for otherwise adequate platelet plugs

#### SUMMARY AND CONCLUSIONS

The histologic appearance of human skin puncture wounds obtained at biopsy, after measurement of the local bleeding times, has been studied in serial section in 3 patients with normal hemostasis and in 4 patients with idiopathic thrombocytopenic purpura It is found that agglutinated platelets arrest hemorrhage in normal skin by rapidly sealing the mouths of all cut vessels larger than capillaries Such

\* Unlike clinical bleeding times these punctures are of the skin of the neck and abdomen

platelet thrombi can resist the effective blood pressure in a cut arteriole of 55 micra. The puncture tract is normally filled with red cell-fibrin clot into which the platelet thrombi protrude. The red clot seals the mouth of the few opened capillaries which can be identified. Other capillaries may be sealed by endothelial agglutination. Fibrin does not enter or form within the injured vessels.

Platelet thrombosis does not occur in idiopathic thrombocytopenic purpura. When larger arterioles and venules are cut the bleeding time is greatly prolonged and fibrin fails to form within the wound because of the speed of blood flow. When smaller vessels are cut in purpura the bleeding time is moderately prolonged, but the cut vessels are eventually sealed by fibrin alone. In thrombocytopenic purpura the bleeding time is normal if only capillaries are cut, since these are normally sealed by fibrin.

The similarity of the histologic appearance of human puncture wounds to that described after experimental vascular injury in other mammals, suggests considerable similarity in mammalian hemostatic mechanisms.

Clinical bleeding time tests vary greatly in depth of puncture and in the caliber and number of the vessels cut. Sufficient volume of hemorrhage during the first minute is thought to be the best guide to an adequate test of the entire hemostatic mechanism.

## REFERENCES

- <sup>1</sup> ZUCKER, M. B. *Am J Phys* 148 275 1947
- <sup>2</sup> HAYEM, G. *Compt Rend Acad Sci Paris* 95 18 1881.
- <sup>3</sup> BIZZAZERO, J. *Virch Arch* 90 261 1881.
- <sup>4</sup> APITZ, K. *Virch Arch* 308 540 1942.
- <sup>5</sup> MACFARLANE, R. G. *Quart J Med* 10 1 1941.
- <sup>6</sup> MAONUI, G. *Arch f klin Chir* 143 574 1926.
- <sup>7</sup> APITZ, K. *Ztschr f d ges exp Med* 111 554 1942.
- <sup>8</sup> DUKE, W. W. *J A M A* 55 1185 1910.
- <sup>9</sup> SPALTEHOLZ, W. *Arch f Anat u Phys (Anat Abt)* 1 54, 1893.
- <sup>10</sup> CHAMBERS, R. AND ZWEIFACH, B. W. *Am J Anat* 75 173 1944.
- <sup>11</sup> SCHIMMELBUSCH, C. *Virch Arch* 101 201, 1885.
- <sup>12</sup> FITZGERALD, P. J., AUERBACH, O., AND FRAME, E. *Blood* 2 519, 1947.
- <sup>13</sup> EBERTH, J. C. AND SCHIMMELBUSCH, C. *Virch Arch* 103 39 1886.
- <sup>14</sup> — AND — *Virch Arch* 105 331 1886.
- <sup>15</sup> WELCH, W. H. *Tr Path Soc Phil* 13 281 1887. Reprinted in *Papers and Addresses by Wm Henry Welch* Baltimore, Johns Hopkins Hospital Press 1920.
- <sup>16</sup> ASCHOFF, L. *Virch Arch* 130 93 1892.
- <sup>17</sup> ZURHELLE, E. *Beitr path Anat* 47 539 1910.
- <sup>18</sup> SCHMIDT, M. B. AND APITZ, K. QUOTED IN SCHMIDT, M. B. *Verh dtsh path Gesell*, 25 10 1930.
- <sup>19</sup> LUBNITZKY, S. *Arch f exp Path u Pharm* 19 185 1885.
- <sup>20</sup> ASCHOFF, L. *Lectures on Pathology* Lecture XI New York, P. B. Hoeber 1924.
- <sup>21</sup> COPLAY, A. L. AND HOULIHAN, R. B. *Blood (Supp)* 1 182 1947.
- <sup>22</sup> TOCANTINS, L. J. *Am J Clin Path* 6 160 1936.
- <sup>23</sup> — *Ann Surg* 125 292 1947.
- <sup>24</sup> QUICK, A. J. *The Hemorrhagic Diseases* Baltimore C. C. Thomas 1942.
- <sup>25</sup> TANNENBERG, J. AND HERRMANN, *Arch f klin Chir* 147 721 1927.
- <sup>26</sup> CLARK, E. R. AND CLARK, E. L. *Am J Anat* 73 215 1943.
- <sup>27</sup> Quoted in M. B. ZUCKER<sup>1</sup>
- <sup>28</sup> MULLER, E. *Beitr z path Anat u z allg Path* 86 273 1931.
- <sup>29</sup> APITZ, K. *Ztschr f d ges exp Med* 111 540 1942.

# THE EFFECT OF THE PARENTERAL INJECTION OF EPINEPHRIN ON LEUKOCYTE COUNTS IN NORMAL SUBJECTS AND IN PATIENTS WITH ADDISON'S DISEASE

B<sub>3</sub> JACQUES L. GABRILOVE, M D, MARIO VOLTERRA, M D, MILDRED D. JACOBS, A B,  
AND LOUIS J. SOFFER, M D

IT HAS been known for many years that the parenteral administration of epinephrin induces marked temporary changes in the white blood cell picture. The mechanism responsible for these changes is still obscure. Interest in this phenomenon, however, has recently been revived since Dougherty and White<sup>1</sup> have demonstrated that the administration of adrenotrophic hormone of the anterior hypophysis or whole adrenal cortical extract results in the destruction of lymphoid tissue with the production of a lymphocytopenia. Long and his group<sup>2</sup> have shown that the injection of epinephrin into experimental animals causes stimulation of the anterior hypophysis with increased secretion of adrenotrophic factor, which in turn stimulates the adrenal cortex. In view of this observation, it was thought desirable to study the effect of epinephrin on the leukocyte count in normal subjects and in patients with Addison's disease, the latter being the closest clinical analogue of the bilaterally adrenalectomized animal.

The lymphocytopenia resulting from adrenal cortical secretion is presumed to be part of the reaction to stress involved in the adaptation syndrome.<sup>3</sup> Pincus et al.<sup>4-5</sup> have used this phenomenon to study reactions to stress in the human as a measure of adrenal cortical secretion.

Conversely, it has long been known, as recently emphasized by De la Balze and co-workers,<sup>6</sup> that patients with Addison's disease have a lymphocytosis associated with a reduction in the total white blood cell count, as well as a decrease in the number of neutrophils.

Previously published studies with epinephrin have almost invariably been short period experiments, usually for less than two hours, since the problem being investigated dealt, for the most part, with the role of the spleen in the ensuing leukocytosis. The administration of epinephrin results initially in a leukocytosis, associated with a sharp increase in the absolute and relative number of lymphocytes. These changes disappear within an hour and are followed by a neutropenia. More recent studies on the effects of epinephrin on the lymphocyte count in normal and adrenalectomized dogs have shown that the former develop a lymphocytopenia which is not observed to occur in the totally adrenalectomized animals.<sup>7</sup>

## METHODS

Ten normal subjects and 11 patients with Addison's disease were studied. Seventy-five hundredths cc of a 1 to 1,000 aqueous epinephrin solution (0.75 milligrams) was administered subcutaneously. Blood for total white blood cell counts and differential studies was obtained from the finger before the administration of epinephrin as well as at fifteen minutes, one hour, two hours, three hours, four hours.

From The Medical Services of The Mount Sinai Hospital, New York.

and five hours following the injection. Smears were stained with Wright's stain and one hundred cells were counted. All tests were performed at approximately the same time of day, from 8 to 9 A.M. to 1 to 2 P.M. Because of the fear of hypoglycemia in the patients with Addison's disease, all subjects were allowed to eat prior to the test and at luncheon time (about 12 noon). All patients experienced palpitation, apprehension, tachycardia and nervousness as the result of the administration of epinephrine.

The diagnosis of Addison's disease had been established in each instance on the basis of adequate clinical and laboratory evidence. All the patients presented the classic clinical picture, and each had been in acute adrenal insufficiency, either occurring spontaneously or induced by salt deprivation on at least one occasion. The characteristic blood electrolyte pattern demonstrating a low serum sodium and chloride and elevation of serum potassium was manifested by every patient of the group selected for study. The members of the group were treated with desoxycorticosterone acetate. None received whole adrenal cortical extract.

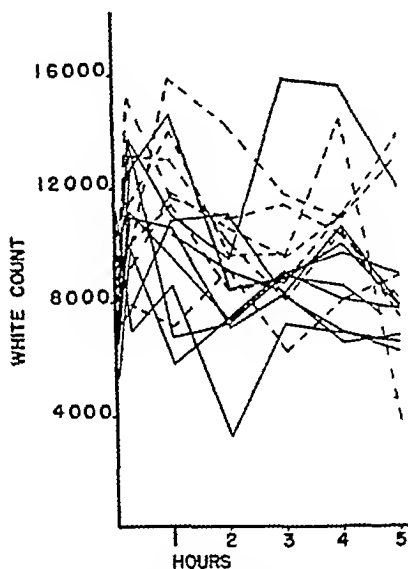


FIG. 1—The total white count following the subcutaneous administration of 0.75 cc. of 1/1000 epinephrine. Broken lines denote normal subjects. Continuous lines denote patients with Addison's disease.

### RESULTS

In both normal subjects and in patients with Addison's disease, the total white blood cell count (fig. 1) exhibits a biphasic character with a high early peak and a low late peak. The peaks occur at fifteen minutes to one hour and at three to four hours. The minimum count is noted at two to three hours. In the normal subjects, as opposed to the patients with adrenal hypofunction, the total white blood cell count is initially higher, and maintains a higher level throughout the test. In addition, in normals, the second peak is much higher than it is in patients with Addison's disease.

In both normal subjects and patients with Addison's disease, the absolute neutrophil count exhibits a biphasic curve. The early peak occurs in fifteen minutes to one hour and is low. The minimum count is noted in one to two hours. The second



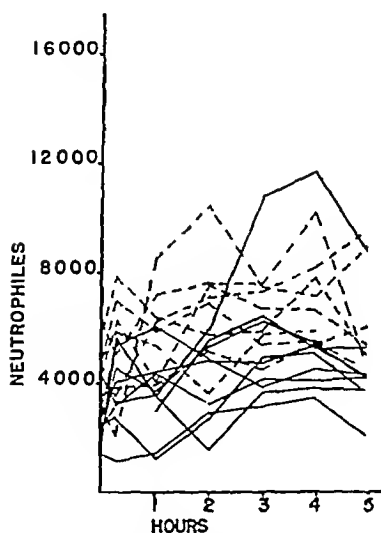


FIG. 2.—The absolute neutrophil count following the subcutaneous administration of 0.75 cc. of  $1/1000$  epinephrine. Broken lines denote normal subjects. Continuous lines denote patients with Addison's disease.

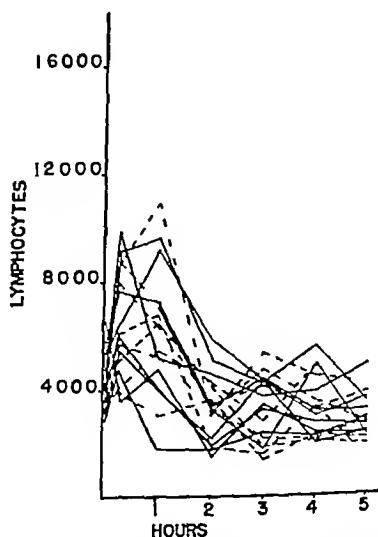


FIG. 3.—The absolute lymphocyte count following the subcutaneous administration of 0.75 cc. of  $1/1000$  epinephrine. Broken lines denote normal subjects. Continuous lines denote patients with Addison's disease.

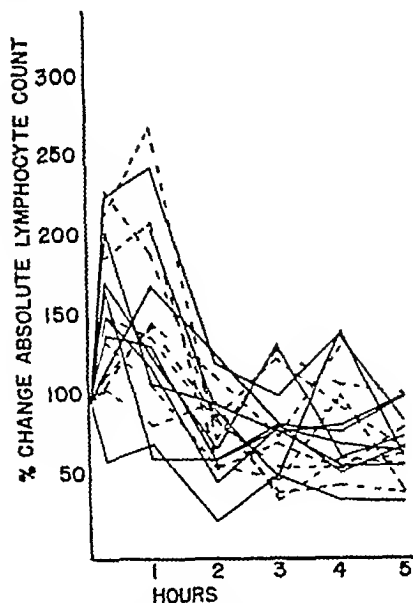


FIG 4—The percentage of the original absolute lymphocyte count following the subcutaneous administration of 0.75 cc of 1/1000 epinephrine. Broken lines denote normal subjects. Continuous lines denote patients with Addison's disease.

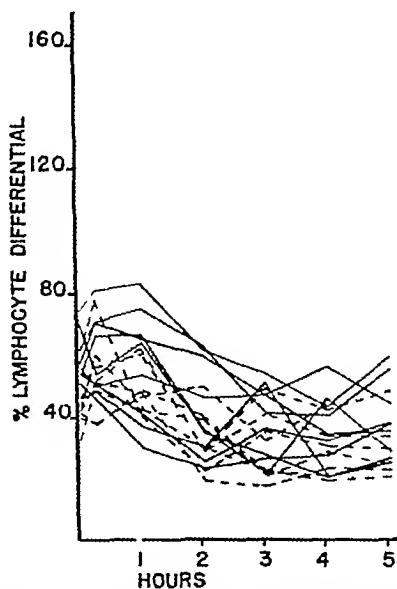


FIG 5—The percentage of lymphocytes in the differential leukocyte count following the subcutaneous administration of 0.75 cc of 1/1000 epinephrine. Broken lines denote normal subjects. Continuous lines denote patients with Addison's disease.

peak is more pronounced than the primary rise, and the second peak in normals is far greater than that noted in patients with adrenal insufficiency. In general, the absolute lymphocyte count in patients with Addison's disease is initially higher than in normals, and maintains this higher level throughout the test. The overlap is so great that no more pronounced lymphocytopenia in normals as contrasted to patients with Addison's disease can be demonstrated as a means of differentiation between the groups (figs. 2 and 3).

When the percentage of the absolute lymphocyte count at any time compared to the original absolute lymphocyte count is plotted against time, a diphasic curve is noted. Here, too, no definite evidence of more marked lymphocytopenia in the normal as opposed to the patient with Addison's disease is noted (fig. 4).

Although we do not feel that the differential count per se in this test is of significance, we have plotted it merely to illustrate how the relative composition of the blood varies. The lymphocyte percentage in the patient with Addison's disease is initially higher than in the normal, and this higher percentage is maintained throughout the test. In both the normals and the patients with Addison's disease, the percentage of lymphocytes rises early and falls late (fig. 5).

With our technic, employing Wright's stain for differential study, no difference in eosinophilia is noted between the two groups. There is a slight late decrease in eosinophilia following the administration of epinephrin.

### DISCUSSION

On the basis of the results obtained, no clear cut separation can be made in the reaction to epinephrin of the patients with Addison's disease from that of the normal individuals.

Several possibilities exist to explain why the expected altered reaction in the patients with Addison's disease as compared to normals was not encountered.

1. Seventy-five hundredths cc. of 1 to 1,000 epinephrin (0.75 mg.) is insufficient to result in stimulation of the anterior pituitary lobe to the secretion of increased amounts of adrenotrophic hormone.

Long et al.,<sup>2</sup> working with animals, employed 0.02 milligrams of epinephrin per 100 grams of body weight. Comparable dosage would necessitate the use of 10 milligrams in a man weighing 50 kilograms. Malmejac et al.,<sup>22</sup> working with dogs, employed 0.1 to 0.2 milligram per kilogram of body weight, intravenously every five minutes for six doses. This is equivalent to 5 to 10 mg. every five minutes in a man weighing 50 kilograms. They found that with this dosage the lymphocytes fell to two-thirds the initial value.

2. The effect is not demonstrable within five hours.

This is rather unlikely, since White and Dougherty<sup>1</sup> demonstrated the effect of adrenotrophic and adrenocortical hormone in one hour, even though the maximum effect occurred in nine hours. Long and Fry<sup>2</sup> produced adrenal changes within two hours after the administration of epinephrin, while Malmejac et al.<sup>22</sup> noted maximum effects in two to three hours.

3. In patients with Addison's disease there is still some responsive adrenal cortical tissue.

It is obvious that all grades of adrenal insufficiency both quantitatively and qualitatively exist. The lymphocyte effect is believed to depend on the carbohydrate regulating fraction of the adrenal cortex. It is possible that in clinical Addison's disease sufficient responding adrenal cortical tissue is present to react following the administration of epinephrin, with some resulting resemblance to the normal reaction.

4. The extraneous effects of epinephrin may mask its effect on the pituitary-adrenal relationship. This possible effect of epinephrin on contraction of the spleen, hemoconcentration, redistribution of formed elements in the blood may interfere with the detection of the effect being studied.

#### SUMMARY

Ten normal subjects and 11 patients with Addison's disease were studied as to their leukocyte response following the subcutaneous administration of epinephrin. The pattern of response was found to be similar in both groups, diphasic curves being noted. In general, the patients with Addison's disease differ from normal individuals in having (1) a lower and less labile white count, (2) a lower and less labile neutrophile count, (3) a higher lymphocyte count, (4) a slightly lesser percentage fall in absolute number of lymphocytes, and (5) a higher lymphocyte percentage.

The use of this method to demonstrate adrenal cortical destruction is not feasible with the dosage of epinephrin employed in this study.

#### REFERENCES

- 1 DOUGHERTY T F AND WHITE A. Influence of hormones on lymphoid tissue structure and function. The role of pituitary adrenotrophic hormone in the regulation of the lymphocytes and other cellular elements of the blood. *Endocrinology* 35: 1 1944.
- 2 LONO C N H AND FRY E G. Effect of epinephrin on adrenal cholesterol and ascorbic acid. *Proc. Soc. Exper. Biol. & Med.* 59: 67 1945.
- 3 SELYE H. The general adaptation syndrome and the diseases of adaptation. *J. Clin. Endocrinol.* 6: 117 1946.
- 4 ELMADJIAN F, AND PINCUS G. The adrenal cortex and the lymphocytopenia of stress. *Endocrinology* 37: 47 1945.
- 5 — AND —. A study of the diurnal variations in circulating lymphocytes in normal and psychotic subjects. *J. Clin. Endocrinol.* 6: 287 1946.
- 6 PINCUS G AND ELMADJIAN F. The lymphocyte response to heat stress in normal and psychotic subjects. *J. Clin. Endocrinol.* 6: 295 1946.
- 7 HOAGLAND H, ELMADJIAN F AND PINCUS G. Stressful psychomotor performance and adrenal cortical function as indicated by the lymphocyte response. *J. Clin. Endocrinol.* 6: 301 1946.
- 8 FREEMAN H AND ELMADJIAN F. The relationship between blood sugar and lymphocyte levels in normal individuals. *J. Clin. Endocrinol.* 6: 668 1946.
- 9 DE LA BALZE F A, REITENSTEIN E C JR, AND ALBRIGHT F. Differential blood counts in certain adrenal cortical disorders (Cushing's syndrome, Addison's disease and panhypopituitarism). *J. Clin. Endocrinol.* 6: 312 1946.
- 10 YANO C S. The influence of adrenalin on the human spleen and blood picture. *Chinese J. Physiol.* 2: 163 1928.
- 11 BILLIOREIMER, ERNST. Ueber die Wirkungsweise der probatotischen Adrenalininjection. *Deutsch. Arch. f. Klin. Med.* 136: 1 1921.
- 12 FREY W. Der Einfluss des Vegetativen Nervensystems auf das Blutbild. *Zeitschr. f. ges. exp. Med.* 2: 38 1913.

- <sup>12</sup> KΛOI, A. Studien und Kritik der Blutveränderung nach Adrenalin *Folia Hemat.* 25 107, 1920
- <sup>14</sup> LOEFER, M. AND CROUZON O. L'action de l'adrénaline sur le sang *Arch de Méd exper* 16 83 1904
- <sup>15</sup> PATEK, A. J., JR. AND DELAND G. A. The effect of adrenalin injections on the blood of patients with and without spleens *Am J M. Sc.* 190 14 1935
- <sup>16</sup> WAKIN, K. G. The effects of adrenalin and nembutal anesthesia on blood constituents before and after splenectomy *J Lab & Clin Med* 31 18 1946
- <sup>17</sup> BENHAMOU E. L'Exploration Fonctionnelle de la Rate Paris Masson & Cie 1933
- <sup>18</sup> HATIEGAN, J. Untersuchungen ueber die Adrenalinwirkung auf die weissen Blutzellen *Wiener Klin Wchnschr* 30 1541 1917
- <sup>19</sup> WALTERHOFER G. Die Veränderungen des weissen Blutbildes nach Adrenalininjection *Deutsch f Klin Med* 135 208, 1921
- <sup>20</sup> SKORCZEWSKI, W., AND WASSERBERG P. Besteht ein Zusammenhang zwischen des Nervus Vagus und der Nervus sympathicus einerseits und der unter der Wirkung spezifischer Gifte veränderten Zusammensetzung des Blutes anderseits? *Ztschr f exper Pathol n Therapie* 10 330 1912
- <sup>21</sup> LUCIA S. P. LEONARD, M. E. AND FALCOVER, E. H. The effect of the subcutaneous injection of adrenalin on the lymphocyte count of splenectomized patients and of patients with certain diseases of the hematopoietic and lymphatic systems *Am J M. Sc* 194 35 1937
- <sup>22</sup> EDMUNDS C. W. AND STONE R. P. The effect of epinephrin upon the number of blood cells *Arch. Int de Pharmacodyn et de Thérap* 28 391 1924
- <sup>23</sup> MALMÉJAC, J. CHARDON G. AND GROS A. Sur la lymphopénie consécutive à l'injection intraveineuse d'adrénaline *Bull Acad Méd* 120 492 1946
- <sup>24</sup> NORDENSON N. G. Clinical experimental observations on the influence of adreno-corticotrophic hormone and desoxycorticosterone (DOCA) on the peripheral blood and the bone marrow *Acta Med Scandinav. Suppl* 196 419 1947

# STUDIES OF BLOOD PASSED THROUGH AN ARTIFICIAL KIDNEY

By NANNIE K M DE LEEUW, M D, AND A BLAUSTEIN, M D

THE USE of the artificial kidney offers a new method of dealing with acute uremia. Many case reports, including some of our own,<sup>1</sup> attest to its value and undoubtedly with time it will be used with greater frequency.

One striking feature is the exteriorization of the patient's blood in an artificial system. The purpose of this investigation was to observe any quantitative or qualitative changes in the formed elements of the blood, that might occur in the use of the artificial kidney. Our studies were *in vitro*.

## MATERIALS AND METHODS

1. The artificial kidney used was the Kolff model.<sup>2-3</sup> Briefly, the blood is directed from the radial artery through a cannula via a system of rubber tubing and a rotating coupling into cellophane tubing approximately 30 meters in length (fig. 1). The latter is wrapped around a rotating drum, which is partially immersed in dialyzing fluid. The blood is then pumped through a Beck pump (fig. 2) which directs it into an airtrap and aids in its return into an antecubital vein.

The dialyzing fluid is heated by an electric element. The machine is provided with varnished metal splashboards, which are partially immersed in the dialyzing fluid.

The principle of the machine is that during the exteriorization of the blood, waste products dialyze out into the surrounding fluid by a process of ultrafiltration.

2. The following influences had to be considered in relation to blood changes:

*Heparin.* In our experiments a concentration of approximately 1 mg. of heparin per 10 cc. of blood was used.

*Cellophane.* The cellophane tubing used was a pure cellulose, to which a small amount of glycerin was added as a softening agent. The cellophane was boiled for ten minutes and thoroughly rinsed with normal saline prior to its use.

*Dialyzing fluid.* The dialyzing fluid consisted of 100 liters of tapwater to which was added 1500 grams of glucose, 600 grams of NaCl, 200 grams of NaHCO<sub>3</sub>, and 40 grams of KCl.

The electrolyte pattern is expressed in the Gamble diagram comparing the blood plasma and the dialyzing fluid (fig. 3). The dialyzing fluid is hypertonic ( $\pm 350$  milli-osmols) as compared with the plasma ( $\pm 310$  milli-osmols).

The temperature of the dialyzing fluid was kept approximately at 100 F.

*Rotation of the drum.* The drum rotated at 26 revolutions per minute.

*Time.* The factor of time was studied in relation to the influence of rotation of the drum and heparinization.\*

*Pump.* The influence of the pump was studied in relation to hemolysis.

*Splashboards.* The influence of the splashboards (galvanized iron and aluminum) on the pH of the dialyzing fluid was investigated in relation to hemolysis.

3. *Errors of methods.* The instruments used were all standardized: Wintrobe hematocrit tubes, Bureau of Standards erythrocyte and leukocyte pipets and a Beckman pH meter. The Rees-Ecker method was used for platelet determination. The coverslip method was chosen for our differential studies.

In our calculations we considered as significant only those erythrocyte counts that differed 12 per cent, leukocyte counts that differed 20 per cent, and platelet counts that differed 30 per cent (in the high counts) and 40 per cent (in the low counts).<sup>4-6</sup>

From the Departments of Surgery and Hematology, Royal Victoria Hospital, Montreal, Quebec, Canada.

This work was supported by a grant from the Archibald Fund.

\* In *in vivo* work it was noted that it took approximately four minutes for the blood to circulate from the arterial end to the venous end of the drum.

In differential counts, 200 leukocytes were counted and the  $\sqrt{n p q}$  theorem was used for standard deviation (according to Plum and Barnett<sup>4</sup>) the maximum error being three times the standard deviation

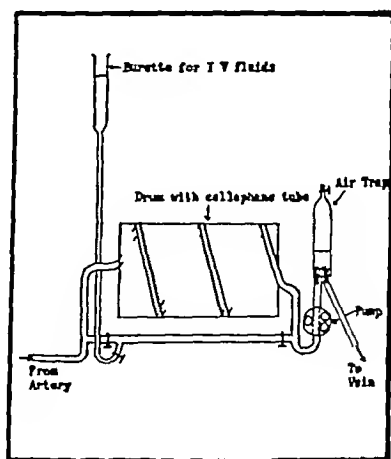


FIG 1 —SCHEMATIC DIAGRAM OF ARTIFICIAL KIDNEY



FIG 2a —BECK PUMP

FIG 2b —BECK PUMP (Fig 34 in De Kunstmatige Nier Profefschrift, Kampen Holland holf W J Permission of the publisher)

According to Wintrobe<sup>7</sup> the technical errors in hematocrit readings on freshly drawn blood are seldom greater than 2.5 per cent. However, in our own experiments it is possible that somewhat larger errors may have occurred as a result of the alterations in size and shape of the red cells which probably occurred during dialysis and rotation.

4. *Materials* Blood samples were taken from 6 patients without blood dyscrasia, 10 healthy nurses and doctors, 10 healthy donors of the blood bank.

## IN VITRO EXPERIMENTS

## Method

The artificial kidney was set up in the manner used for *in vivo* work. Approximately 20-30 cc of blood was taken from a volunteer, 2-3 mg of heparin was placed in the syringe prior to the venipuncture. Divided samples were then used. Samples of the heparinized blood were kept in test tubes and initial blood studies were carried out (table 1, column I), and determinations repeated after 30-60 minutes (column II). Samples of the same blood were placed in segments of cellophane tubing, 50 cm in length, which were then closed at both ends and suspended in the dialyzing fluid for 15-25 minutes (column III). Other segments of cellophane tubing of the same length were filled with approximately 10 cc of

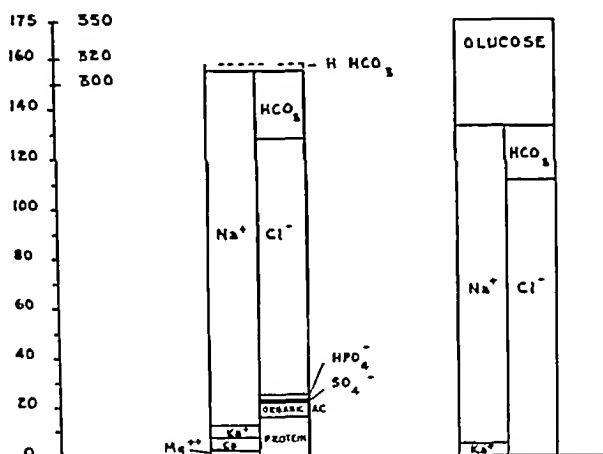


FIG 3—COMPARISON OF BLOOD PLASMA (LEFT) AND DIALYZING FLUID (Chart 34 in *Extracellular Fluid* by J. L. Gamble Cambridge 1942. Permission of the author)

blood, attached to the drum and rotated for 3-8 minutes (column IV), and 15-25 minutes (column V).

We were able to observe the influence of rotation of the drum by comparing the results of the rotated samples with those obtained from the suspended blood samples. By further comparing the last findings with those of the control samples of heparinized blood the effect of the dialyzing fluid, the cellophane and the rinsing fluid was determined. Comparison of the initial and the second determinations on the control samples allowed us to surmise the possible effect of heparin on the formed blood elements. Our studies were confined to red blood cell counts, hematocrit readings, white blood cell counts, platelet determinations and differential counts of the leukocytes.

The results of these determinations are summarized in table 1.

*Interpretation* Comparing columns I and V significant changes were found in hematocrit readings, but only in two red blood cell counts. There was a significant



change in all leukocyte and platelet counts. A great number of factors could account for these changes, and therefore we made the following differentiation

TABLE I

Name	Sex	Condition	Column I	Column II	Column III	Column IV	Column V
			Samples				
			Initial	Control 30-60 min	Suspended 15-25 min	Rotated 3-8 min	Rotated 15-25 min.
			Influences				
			heparin	heparin, time	heparin time dialyzing fluid, rinsing fluid cellophane	heparin time dialyzing fluid, rinsing fluid, cellophane rotation	heparin, time dialyzing fluid, rinsing fluid, cellophane rotation

## Hematocrit

B. W.	M.	N. H.	57	57 (40)	54.5 (18)	54.5 (3)	52.3 (15)
A. B.	M.	N. H.	52.3	52.3 (30)	49 (17)	47.9 (3)	43.6 (15)
N. L.	F.	N. H.	49	47.9 (30)	49 (15)	47.9 (3)	46.8 (15)
C. N.	F.	N. H.	43.6	43.6 (30)	43.6 (15)	43.6 (3)	42.4 (15)
R. B.	M.	Postop. poloid.	49	49 (60)		43.6 (8)	42.5 (25)

## Red Blood Cells (in mill)

B. W.			5.70	5.34 (40)	5.00 (18)	5.25 (3)	4.99 (15)
A. B.			5.04	5.02 (30)	5.01 (17)	4.36 (3)	4.42 (15)
N. L.			4.87	4.82 (30)	4.37 (15)	4.46 (3)	4.38 (15)
C. N.			4.60	4.41 (30)	4.12 (15)	4.08 (3)	4.10 (15)
R. B.			4.82	4.89 (60)		4.28 (8)	4.27 (25)
L. F.	M.	Chron. thick. R ankle	5.18	5.10 (30)	5.14 (22)	5.22 (5)	4.70 (22)

## White Blood Cells

B. W.			7500	7750 (40)	7200 (18)	5800 (3)	5400 (15)
A. B.			7200	7050 (30)	6250 (17)	5150 (3)	4950 (15)
N. L.			7050	7250 (30)	7000 (15)	7450 (3)	5450 (15)
C. N.			8350	8150 (30)	7800 (15)	6000 (3)	3550 (15)
R. B.			8250	8300 (60)		6600 (8)	5150 (25)
L. F.			6000	6050 (30)	4875 (22)	2800 (3)	1150 (22)

## Platelets (in thousands)

B. W.			890	610 (40)	460 (18)	380 (3)	180 (15)
A. B.			610	520 (30)	380 (17)	410 (3)	160 (15)
N. L.			200	190 (30)	190 (15)	200 (3)	110 (15)
C. N.			240	190 (30)	180 (15)	70 (3)	60 (15)
R. B.			620	610 (60)		450 (8)	290 (25)

Italic figures = Significant difference. ( ) = time in min.

i. Comparison of columns I and II allows for the interpretation of the influence of heparin for a certain period of time. No significant changes were found in hemato-

crit readings (with one exception), red blood cell counts, white blood cell counts and platelets after thirty minutes

2. Comparing columns III and V, from which the effect of *rotation* of the drum on the blood samples can be determined, a significant drop in the leukocyte and platelet count was found. A slight change in hematocrit determinations was noted, but not in the red cell counts

3. Comparing columns IV and V, in which the influence of the *time* factor on rotation of the drum can be determined, slight changes were found in the hematocrit determinations, but not in the erythrocyte counts. A decided drop in the leukocyte and platelet counts was evident

4. Comparison of the columns II and III allows for the interpretation of the influence of *dialyzing fluid*, *cellophane* and *rinsing material*. In 2 cases, there was a drop in the hematocrit value. There was no significant change in the erythrocyte, leukocyte or platelet counts

5. Comparing columns III and IV, it is apparent that the leukocyte count dropped only in two blood samples through the influence of rotation for a period of three minutes

6. Studies of differential smears were made in order to observe whether the drop in the leukocyte count, noted in column V, was uniform. The smears in all cases, revealing a normal differential picture, did not significantly change in the different blood samples. In the smears made from the blood samples of columns III, IV and V condensation of the nuclei and vacuolization of the cytoplasm with ameboid cell outlines were seen in the granulocytes. Most of the monocytes were vacuolated. The number of hypersegmented polymorphonuclear leukocytes in column V ranged from 7-15 per cent of the total leukocyte count in three out of six cases

*Conclusions* Studies of our control samples reveal that heparin does not exert any significant influence on the erythrocyte, leukocyte and platelet counts in a thirty minute period at room temperature. Rotation of the drum for a period of three minutes sometimes results in a significant drop in the leukocyte count, after a fifteen minute period a decided drop in the leukocyte and platelet count becomes evident. The dialyzing fluid and the rinsing material appear to cause some dilution, as seen in the decrease in hematocrit values after suspension, but more marked after rotation of blood samples in cellophane tubing

#### *The Effect of Dry Cellophane on in Vitro Dialysis*

Segments of cellophane tubing, 50 cm. in length, were boiled, rinsed with saline, dried, and then 5 cc. of heparinized blood was placed in each. Some segments of cellophane tubing filled with heparinized blood were suspended in the dialyzing fluid, others were rotated on the drum for a certain period of time. Results show (table 2) that hemoconcentration or hemodilution may occur, if samples of dry cellophane tubing are used in in vitro dialysis

#### *The Chemotactic Influence of Cellophane on Granulocytes*

In order to investigate the possible chemotactic influence of cellophane on the leukocytes, white blood cell counts were done on samples of heparinized blood,

placed in sterile test tubes in which sterilized strips of cellophane were immersed for a period of one hour, and the results compared with the counts obtained from control samples of blood

*Procedure* Strips of cellophane, 10 cm in length, were boiled, rinsed with large amounts of normal saline, and were dried and sterilized 15 cc of blood was taken under sterile conditions from a donor and divided among three sterile test tubes, each containing 15 mg of heparin Test tube I was used as a control and did not contain cellophane A strip of the sterilized, dried cellophane was placed in test tube II The blood in test tube III was used for initial hematologic determinations Test tubes I and II were stoppered and immersed in a waterbath of approximately 100 F, and white blood counts were done one hour later The results are shown in

TABLE 2.—Hemoconcentration and hemodilution using dry cellophane

Name	Sex	Condition	Cellophane tubing		Temperature dial fluid, F°	Hematocrit value	
			suspended	rotated		test	control
			min	min			
B W	M.	N H.		15	77-80	50	48
P P	M.	Postapp		15	83-87	51	46
I H.	F	N H	20		96-107	38	42
				18	96-101	31	42
N L.	F	N H	15		91-96	39	40
				15	92-96	33	40

TABLE 3

	Name	Sex	Condition	Leukocyte counts after one hour		Initial count III
				I (Control)	II (+ cellophane)	
(α)	S C.	M.	N H.	5,700	5,550	6,200
(β)	P L.	M	N H	4,950	3,050	4,250
(γ)	F C.	M.	N H.	3,950	4,000	6,750

table 3 Differential counts done on samples (β) and (γ) showed no significant changes after one hour as compared with the initial counts

In tests (β) and (γ), two strips of cellophane were immersed in the tubes (II) In test (γ), the heparin concentration was twice as high as in the other tests, which might be responsible for the greater drop of the leukocytes in both tubes

Segments of cellophane, taken after one hour, and stained with Wright's stain, were coated with leukocytes A control piece of cellophane, taken after ten minutes immersion in the blood, showed only a few leukocytes (figs 4a and b (control))

*Conclusion* A definite drop of leukocytes can be seen in two of the three blood samples in which cellophane strips have been immersed for one hour, and in one of the control blood samples after one hour Therefore, we cannot conclude from the white blood counts, that cellophane has a positive chemotactic influence on

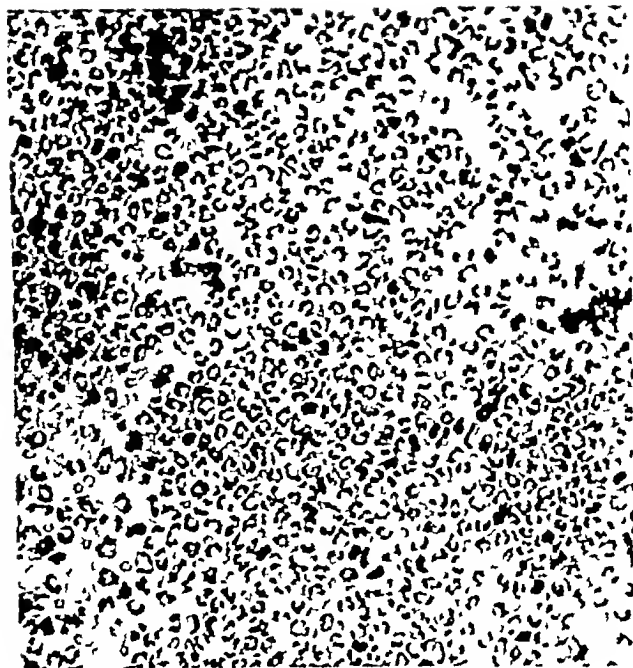


FIG 4a —CHEMOTACTIC INFLUENCE OF CELLOPHANE ON GRANULOCYTES See TEXT

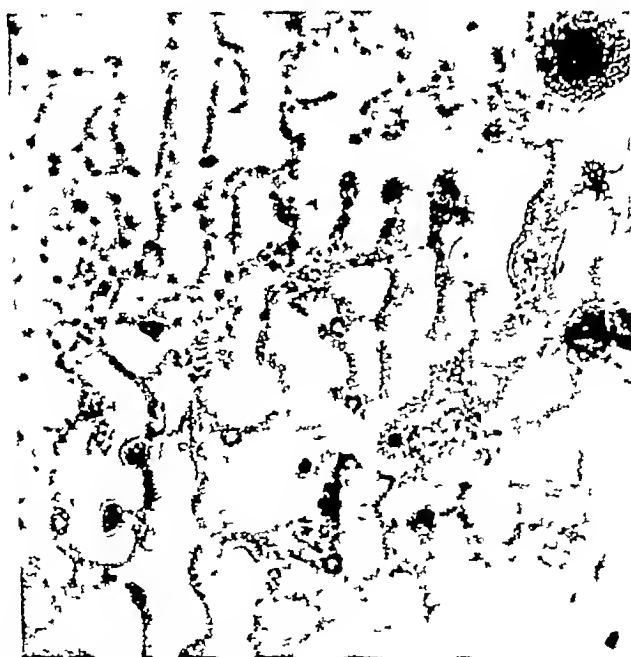


FIG 4b —CHEMOTACTIC INFLUENCE OF CELLOPHANE ON GRANULOCYTES See TEXT.

the leukocytes. However, the vast number of granulocytes present on the sterile strips of cellophane is at least indirect evidence of an attractive influence exerted by the cellophane, almost exclusively for the granulocytes.

### *The Influence of Heparin and Cellophane on the Platelets*

The same test was done as the one just described, but under unsterile conditions, and platelet counts were made from tubes I and II (without and with cellophane) at different intervals (see table 4). It can be seen that the counts dropped equally in both tubes. In the stained smears the platelets were clumped and appeared swollen and disintegrated in the one and two hour samples, and were diminished in number. After two hours the cellophane strips showed large clumps of platelets.

*Conclusion* This concentration of heparin (approximately 1 mg./10 cc. of blood) cannot prevent platelet agglutination. Disintegration of the platelets starts after about half an hour.

*Note* There were also clumps of platelets to be seen on the control strips of cellophane after 10 minutes in the preceding test (chemotactic influence of cellophane on granulocytes).

TABLE 4

Name	Sex	Cond.	Platelet counts		Time
			tube I (hep. blood)	tube II (hep. blood + strip cellophane)	
A. W.	F.	N. H.	300,000	300,000	0
			210,000	180,000	30
			180,000	190,000	60
			100,000	90,000	120

### *Hemolysis*

Hemolysis was commonly noted in all samples of blood subjected to the rotation of the drum. Hemolysis being an important feature, it was decided to investigate possible causes.

### *The Role of Cellophane*

1. Twelve test tubes, each containing 5 cc. of heparinized blood, were set up in a rack. Four were used as controls and did not contain cellophane. Four contained strips of cellophane that had not been boiled or rinsed, and four contained strips of cellophane that had been boiled and rinsed twice with saline and then dried. All tubes were allowed to stay at room temperature for thirty minutes. The control tubes showed no hemolysis, the tubes containing unwashed cellophane showed hemolysis, varying from a trace to one plus, those containing the washed cellophane all showed a trace of hemolysis. Fresh smears of all of these samples did not reveal spherocytosis (table 5).

2 Cellophane tubing, 300 cm in length, was boiled and rinsed with 10 liters of normal saline. Segments, 30 cm in length, were cut and filled with 5 cc of heparinized blood each. These segments were then suspended in a bath of saline at 84-89 F for half an hour. The above was repeated, using segments of cellophane

TABLE 5

	Hemolysis				Spherocytosis			
	Test tubes number							
	1	2	3	4	1	2	3	4
Control blood	no	no	no	no	no	no	no	no
Blood + strip of cellophane	+	sl	tr	sl	no	no	no	no
Blood + strip of washed cellophane	sl	?	tr	tr	no	no	no	no

TABLE 6

Name	Sex	Cond.	Sample	Hemolysis			
				Blood in carefully washed cellophane suspended in normal saline	Blood in unwashed cellophane	Control	
						before	after
W. H.	M.	N. H.	1	no	slight	no	
			2	no	+	no	no
			3	no	+	no	
A. A.	M.	N. H.	4	no	slight	no	
			5	no	slight	no	no
			6	no	slight	no	
E. A.	M.	N. H.	7	no	trace	no	no
			8	no	slight	no	
			9	no	slight	no	
R. A.	M.	N. H.	10	no	+	no	?
			11	no	+	no	
			12	no	slight	no	

Name	Sex	Cond.	Sample	Blood in carefully washed cellophane, suspended in dialyzing fluid	Control	
					before	after
H. D.	M.	N. H.	1	no	no	no
J. R.	M.	N. H.	2	trace	no	no
J. T.	M.	N. H.	3	no	no	no

that were not rinsed with saline. The latter samples of blood revealed a slight to marked hemolysis in all cases, whereas the former showed no hemolysis at all (table 6).

3 Similar experiments, suspending three samples in the dialyzing fluid rather than in saline, did not show any hemolysis in two of these cases. There was a trace of hemolysis in the third case (table 6).

*Role of the Dialyzing Fluid*

Heparinized blood, 5 cc, was added to two test tubes each containing 1 cc of dialyzing fluid. No hemolysis or spherocytosis was to be seen after two hours.

*The Factors of Time, Rotation of the Drum, Length of Cellophane Tubing, Amount of Blood and Temperature of the Dialyzing Fluid*

Samples of blood were placed in cellophane tubing, which was boiled, rinsed with 10 liters of saline and dried prior to its use. The segments were then attached to the drum. The above factors were all varied and their influences studied (table 7). Hemolysis occurred in all samples of blood rotated on the drum. In two of the four samples, hemolysis was more marked after rotation for fifteen minutes rather

TABLE 7—Hemolysis in segments of carefully washed cellophane, rotated on the drum

Name	Sex	Cond	Temp dial fluid	Length cellophane			Control	
				80 cm.	80 cm	40 cm		
				Amount blood				
				5 cc.	5 cc.	20 cc.		
				Time rotation			before	After
4 min	15 min	4 min						
A. P	M	N H	96- 97	+	+		no	no
H. D	M	N H	97-101	+	++	slight	no	no
R. B	M	N H.	97-101	+	++	trace	no	no
J R.	M	N H.	97-101	+	+	slight	no	no
J T	M	N H.	90	++			no	no
			109	+			no	no

than three minutes. Less hemolysis occurred when the cellophane tubing contained a larger amount of blood. In two cases studied, there was less hemolysis with increase of temperature of the dialyzing fluid (109 F) as compared with samples rotating at 90 F.

*Role of the Pump*

Samples (5 cc) of heparinized blood were placed in a circuit of rubber tubing and sent through the pump for periods of one and three minutes. Samples were then centrifuged in test tubes and in all cases there was slight hemolysis. No changes were found in hematocrit determinations, erythrocyte, leukocyte and platelet counts after three minutes (table 8).

*Handling of the Cellophane*

Experiments were carried out to determine whether the handling of the cellophane containing the blood influenced the occurrence of hemolysis. Hemolysis appeared to be more marked in those samples of blood that were handled

*pH of the Dialyzing Fluid*

Determinations of the pH of the dialyzing fluid were made before and after experimental use of the artificial kidney. The pH increased after *in vitro* use of the artificial kidney for about six hours (table 9). It was thought that the metal splashboards might alter the pH of the dialyzing fluid, and pH determinations were carried out in experiments where the splashboards were not used. It appeared that the immersion of these in the dialyzing fluid resulted in a rise in the pH (table 9).

TABLE 8 — *Influence of the Pump on the Blood*

Name	Sex	Condition	Hemolysis			Case L. F.	Control	Pump 3 min
			Control	Pump 1 mn	Pump 3 min			
L. F.	M	skingraft	no		slight	Hematocrit	46	46
M. T.	F	N. H.	no		slight	Erythrocytes	5 mill	5 mill
M. M.	F	N. H.	no		slight	Leukocytes	7,200	7,400
P. P.	M	postappend	no	slight		Platelets	610,000	580,000
N. L.	F	N. H.	no	trace	slight			

TABLE 9

Test	pH of dialyzing fluid			Time approx	Temp. C°
	Before use	After use no splash boards	After use + splashboards		
1			8.85	6 hr	20.5
2	8.35		8.7	6 hr	20.3
3	8.35		8.8	6 hr	24
4	8.15	8.37 (1 hr)	8.58	3 hr	25

## DISCUSSION

*Erythrocytes*

Some dilution of the blood occurs as a result of the passage of the dialyzing fluid through the cellophane in suspended and in rotated blood samples. The possible presence of some of the rinsing fluid in the cellophane tubing may also account for some of the dilution which is evident through the decreased hematocrit values. The discrepancy between hematocrit and red blood cell values may be related to the shrinkage of the cells in hypertonic medium.

*Leukocytes*

Wilander,<sup>8</sup> Jorpes,<sup>9</sup> and Lucia and Aggeler<sup>10</sup> noted that heparin causes *in vitro* agglutination and disintegration of leukocytes after one hour. In some of our *in vitro* studies using heparinized blood, we were able to observe this phenomenon in our two hour blood samples. No change could be found after half an hour. In our experiments in which samples of blood were suspended in the dialyzing fluid



or rotated on the drum the time never exceeded 15-25 minutes, and therefore we were not able to judge the role of heparin in these experiments

The cellophane used was a pure cellulose. Cellulose, according to Chambers and Grand,<sup>11</sup> exerts positive chemotaxis on granulocytes. We have demonstrated that strips of cellophane suspended in sterile test tubes containing heparinized blood become thickly coated with granulocytes after one hour. The total white blood counts and the differential counts of those blood samples were not significantly altered as compared with control samples of blood, but this is probably related to the fact that chemotaxis does not occur beyond a distance greater than one millimeter, so that with a sample of blood in a test tube no appreciable change in the above values is to be expected.

In our samples of blood that were rotated on the drum, the time factor is important, since the period did not exceed twenty-five minutes in any of these experiments, and, according to Dixon and McCutcheon,<sup>12, 13</sup> it takes at least thirty minutes for the leukocytes to develop their normal rate of locomotion. Stained sections of cellophane from the rotated samples of blood did not reveal an increase of granulocytes. From our chemical determinations we know that glucose crosses the cellophane barrier and enters the blood (samples of kidney blood contain approximately 1400 mg per cent glucose). Chambers and Grand<sup>11</sup> have demonstrated the positive chemotactic influence of glucose on the granulocytes. The granulocytes, therefore, may be in a position of *embarras du choix*.

*Rotation of the drum.* Dilution and the damaging action of slow speed centrifugation probably account for the drop in the leukocytes in the samples of blood rotated on the drum, a drop which is increased with time.

Hypersegmentation of the polymorphonuclear leukocytes was frequently seen in those samples that rotated on the drum for fifteen minutes. Oria<sup>14, 15</sup> has observed hypersegmentation in oxalated and centrifugated blood samples and Ponder<sup>16</sup> in heparinized blood that has been exteriorized for some time. Exteriorization and slow centrifugation may have played a role in our cases.

### *Platelets*

The drop in the platelet count in the suspended samples of blood and in the samples rotated on the drum can be accounted for by dilution, the presence of rough surfaces offered by the cellophane tubing, and the mechanical damage of rotation on the drum. Solandt and Best,<sup>17</sup> Baronofsky and Quick<sup>18</sup> state that large doses of heparin prevent platelet agglutination. The concentration of heparin we used fell below these requirements.

### *Hemolysis*

This phenomenon is of clinical importance in the use of the artificial kidney. Heparin and the dialyzing fluid have no hemolytic action. The cellophane used was a pure cellulose, but had glycerin added as a softening agent. Our experiments proved to us that the cellophane had to be thoroughly rinsed with at least 10 liters of normal saline prior to its use, as advised by Kolff<sup>2, 3</sup> in order to wash away the glycerin which is known to be hemolytic even in small quantities (van Noordwijk<sup>2, 3</sup>).

Rotation of the drum causes hemolysis within four minutes, and the degree of hemolysis increases with time. Handling of the cellophane tubing containing blood demonstrates hemolysis. Blood, subjected to the influence of the pump used in aiding venous return, demonstrated hemolysis within the period of one minute. According to our calculations, during an *in vivo* dialysis of six hours each cc of blood is compressed approximately forty-five times by the rollers of the pump. This is the degree of mechanical injury to which 1 cc of blood was subjected for one minute during our test (each cc being compressed forty-five times by the rollers of the pump during the period of one minute). Therefore, in all probability, during *in vivo* dialysis the action of the pump partly accounts for the occurrence of hemolysis, although it is realized that the studies of hemolysis in relation to the pump are not entirely comparable with the role of the pump in *in vivo* dialysis. The role of high temperature as a cause of hemolysis is well known.<sup>4</sup> In our experiments we did not elevate the temperature of the dialyzing fluid above 109 F, and at that level we did not notice any increased hemolysis.

According to Ponder<sup>4</sup> and Gordon,<sup>19</sup> an extremely high pH and an extremely low pH will act directly on the red cell giving hemolysis. The pH of our dialyzing fluid at no time reached these levels. The metal splashboards may have contributed in altering the pH of the dialyzing fluid. The varnish does not last very long and therefore appears to be no protection against the ionization from the splashboards. The metals used (Zn and Al) do not belong to the group which has hemolytic action in high dilutions.

Kolff<sup>2, 3</sup> demonstrated that rapid rotation of the drum (at 35 revolutions per minute) produces hemolysis. We used a speed of 26 revolutions per minute and also observed hemolysis. Glucose 1.5 per cent added to the dialyzing fluid could not prevent hemolysis in rotating blood samples.

#### *Measures to Minimize Hemolysis*

The cellophane tubing should be boiled in a large amount of water and then carefully rinsed with 10 liters of sterile saline in order to remove the glycerin.

Glucose, 1.5-2 per cent, should be added to the dialyzing fluid in order to inhibit hemolysis.<sup>2, 3</sup>

Alcohol or ether should not be used in mounting the artificial kidney.

Do not use soap in cleaning the enamel bath.

The temperature of the dialyzing fluid should be approximately 100 F.

Handling of the cellophane tubing with the flat hand, in order to push the blood forward, should be avoided.

Avoid unnecessary rotation of the drum during *in vivo* dialysis.

Splashboards should be made of nonhemolytic material. Metal should not be used.

#### SUMMARY

1. *In vitro* blood studies using the artificial kidney are discussed in detail.

2. *In vitro* experiments in which samples of blood in cellophane tubing are rotated on the drum showed a diminution in the red cell volume, leukocyte and platelet counts.

Dilution, caused by the passage of the dialyzing fluid through the cellophane membrane and by the presence of some of the rinsing fluid, and rotation of the drum are factors of importance in this regard

3 The influence of heparin and the chemotactic role of cellophane on the leukocytes are discussed

Heparin in concentration of 1 mg / 10 cc of blood causes in vitro agglutination and disintegration of leukocytes after approximately one hour

Photographs are shown that demonstrate coating of the cellophane by granulocytes This is in keeping with the findings of Chambers and Grand that cellophane exerts a positive chemotactic influence on the granulocytes

4 Hypersegmentation of the leukocytes was noted in samples of blood rotated on the drum for fifteen minutes Exteriorization and slow speed centrifugation probably account for this phenomenon

#### REFERENCES

- <sup>1</sup> MACLEAN J T RIPPSTEIN C B, DE LEEUW N A M AND MILLER G GAVIN *Canad M A J*, 58 433 1948
- <sup>2</sup> KOLFF W J *New Ways of Treating Uremia* London J and A Churchill 1947
- <sup>3</sup> ——— *De kunstmatige nier Kampen Holland Ac Proefschrift* 1946
- <sup>4</sup> PONDER E *The mammalian red cell and the properties of hemolytic systems* Berlin Protoplasma Monografien 1934
- <sup>5</sup> PLUM P *Acta Med Scand* 90 342, 1936
- <sup>6</sup> STUROSIS C S and Bethell F H *Physiol Rev* 23 279 1943
- <sup>7</sup> WINTROBE M M *Arch Int Med* 54 256 1934
- <sup>8</sup> WILANDER O *Acta Med Scand* 94 258 1938
- <sup>9</sup> JORPES J E *Heparin in the treatment of thrombosis* London Oxford University Press, 1946
- <sup>10</sup> LUCIA S P AND AOOELER P M *Proc Soc Exper Biol & Med* 40 41 1939
- <sup>11</sup> CHAMBERS R AND GRAND C G *J Cell & Comp Physiol* 8 1 1936
- <sup>12</sup> DIXON, H M, AND McCUTCHEON M *Proc Soc Exper Biol & Med* 34 173 1936
- <sup>13</sup> McCUTCHEON M *Physiol Rev* 26 319 1946
- <sup>14</sup> ORIA, J *Blood* 3 225 1948
- <sup>15</sup> ——— *Brasil medico* 59 nos 16 and 77 April 1945
- <sup>16</sup> PONDER E J *Lab & Clin Med* 27 866 1942
- <sup>17</sup> SOLANDT D Y AND BEST C H *Lancet* 1 1042 1940
- <sup>18</sup> BARONOFKY I D AND QUICK A J *Proc Soc Exper Biol & Med* 53 173 1943
- <sup>19</sup> GORDON A S *Quart J Exper Med* 22 399 1933

# A METHOD FOR ISOLATING LIVING POLYMORPHONUCLEAR LEUKOCYTES FROM PERIPHERAL BLOOD

By ALLEN H. MINOR, M.D.,\* AND LEE BURNETT, M.S.

THE LIVING polymorphonuclear leukocyte can readily be obtained without significant trauma either to the cell or to its donor. The movement of its granules, as seen with darkfield illumination<sup>1</sup> or supravital staining,<sup>2</sup> affords a visible index of its vitality. These attributes render this cell particularly suitable for cytologic studies. In order to facilitate such studies, we have developed a method for isolating living polymorphonuclear leukocytes on a microscope slide. This method takes advantage of two properties inherent in blood components: the ability of fibrinogen to accelerate erythrocyte sedimentation,<sup>3</sup> and the adhesive quality of living polymorphonuclear leukocytes.<sup>4</sup>

## METHOD

The method used for cell isolation consists of two steps. In the first step, the ratio of erythrocytes to leukocytes is reduced from about 800:1 in normal blood to nearly 1:1. This is accomplished by the addition of fibrinogen to heparinized blood. By this means the erythrocyte sedimentation rate is so accelerated that within an hour essentially all the erythrocytes have settled out, leaving essentially all the leukocytes suspended in the supernatant plasma.<sup>5</sup>

In the second step, polymorphonuclear leukocytes are isolated on a slide from all other cells in the plasma suspension. This is accomplished by means of the adhesiveness of these cells to a solid surface. When the suspension is placed in a vessel similar to that described by Fenn,<sup>6</sup> consisting of a glass ring on a microscope slide, and the blood cells are permitted to sediment, subsequent washing of the slide removes the plasma and all cells except adherent polymorphonuclear leukocytes.

The following technic for this step has been found practical:

1. Place one or more glass rings 3 mm. high by 15 mm. internal diameter on a microscope slide. Press modelling clay against the angle formed by the slide with the exterior of the rings. The clay prevents leakage, appears to be nontoxic, and subsequently may easily be removed from the slide.

2. Pipet 0.3 ml. leukocyte suspension into each of the vessels so formed, cover with a glass slide or coverslip, and incubate at 37°C. for one hour. Suspensions in which the concentration of polymorphonuclear leukocytes is considerably greater than normal may first be diluted with plasma.

3. Immerse the vessels in a 250 ml. beaker filled with normal saline or other physiological solution at room temperature. Remove the rings, taking care to avoid contact with the spots of cells on the slide. Then move the slide back and forth in the fluid a few times. This removes nonadherent cells from the slide, leaving only polymorphonuclear leukocytes.

This work was begun at The Sloan Kettering Institute for Cancer Research, New York, N. Y., and completed at The Lenox Hill Hospital, New York, N. Y.

\* Postdoctorate Research Fellow, National Cancer Institute.

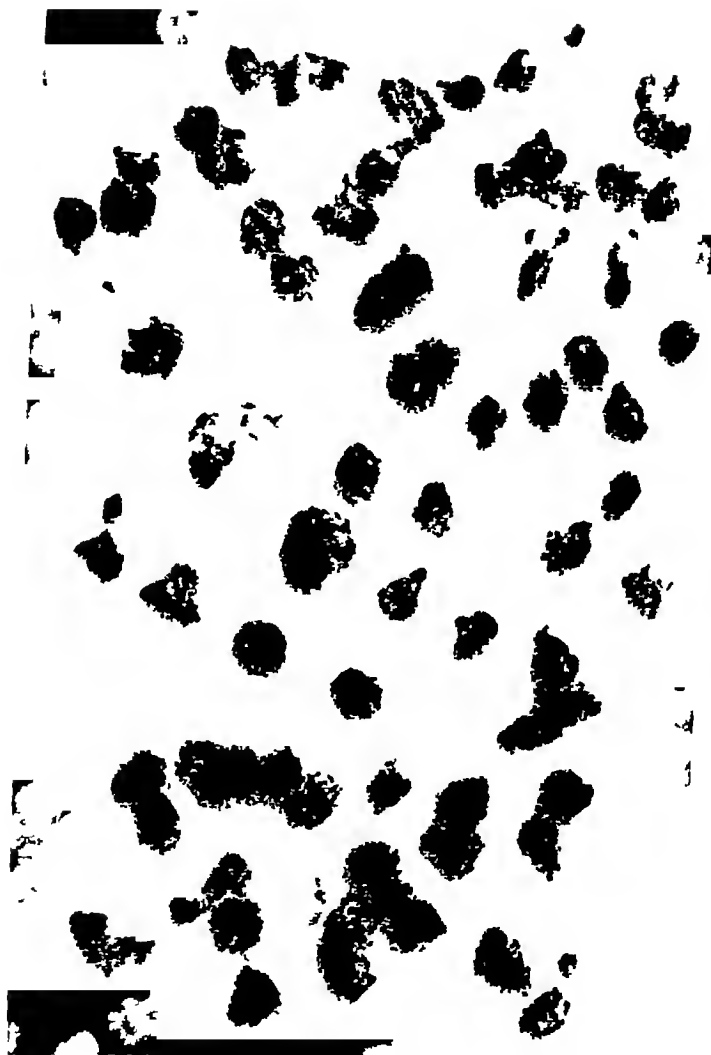


FIG 1—LIVING POLYMORPHONUCLEAR LEUKOCYTES ISOLATED FROM HUMAN PERIPHERAL BLOOD  
X 630 Darkfield illumination

#### DISCUSSION

This method for isolating polymorphonuclear leukocytes may be useful in cell studies for the following reasons

The cells obtained are of a single type This is apparent from the photomicrograph (fig 1) Reactions between these cells and their medium may be observed without the presence of other cell types

The cells are alive, and their vitality is immediately manifest. In a favorable environment, living polymorphonuclear leukocytes exhibit intracellular granular motion and amoeboid movement. These activities are indicated in the photomicrograph by blurring of the granules and irregularity of cell shape. Dynamic changes may thus be observed directly and recorded photographically.

Homologous cells may be compared. Two or more spots of cells may be prepared on a single slide. Comparisons may be made either between cells from different sources exposed to a given chemical or physical agent, or between cells from the same source exposed to different agents. Either the reactive capacities or the chemical composition of the cells may be studied.

The method is technically simple. It utilizes equipment and reagents which are readily available. There is minimal traumatization to the cells.

#### SUMMARY

A simple method is described for isolating living polymorphonuclear leukocytes from peripheral blood. It is based on the selective adherence of these cells to a microscope slide, which permits all other blood components to be removed by washing. The reasons for considering this method useful in cell studies are discussed.

#### ACKNOWLEDGMENT

The authors are indebted to Mr. Martin Shames and the King Weese Studios, New York, for the photomicrograph.

#### REFERENCES

- <sup>1</sup>HANSEN PRUSS, O. C. The circulating blood cells as seen by dark ground illumination. *Am J Clin Path* 6: 423-431, 1936.
- <sup>2</sup>SABIN, F. R. Studies of living human blood cells. *Bull Johns Hopkins Hosp* 34: 277-288, 1923.
- <sup>3</sup>FABRAEUS, R. The suspension stability of the blood. *Physiol Rev* 9: 241-274, 1929.
- <sup>4</sup>EWING, J. *Clinical Pathology of the Blood*. Philadelphia: Lea, 1901. P. 106.
- <sup>5</sup>MINOR, A. H., AND BURNETT, L. A method for obtaining living leukocytes from human peripheral blood by acceleration of erythrocyte sedimentation. *Blood* 3: 799-802, 1948.
- <sup>6</sup>FENN, W. O. The adhesiveness of leucocytes to solid surfaces. *J Gen Physiol* 5: 143-167, 1922-23.

# A METHOD FOR THE RAPID SEPARATION OF LEUKOCYTES AND NUCLEATED ERYTHROCYTES FROM BLOOD OR MARROW WITH A PHYTOHEMAGGLUTININ FROM RED BEANS (*PHASEOLUS VULGARIS*)

By JONAH G. LI, M.D., AND EDWIN E. OSGOOD, M.D.

A TECHNICALLY simple and rapid method for separating living leukocytes and nucleated erythrocytes from whole blood or marrow with a high degree of efficiency, large net yield and negligible admixture with mature erythrocytes or other contaminants is needed for chemical, metabolic or cultural studies of these cells. The method presented in this paper describes a technic for accelerating the sedimentation of erythrocytes, leaving unaltered leukocytes and other nucleated cells suspended in their own plasma. This separation can be accomplished in approximately ten minutes from the time the blood or marrow specimen has been collected. It is applicable to any volume of blood from less than 10 ml. to over 500 ml.

## METHOD

*Principle* Erythrocytes are agglutinated by addition of the phytohemagglutinin, sedimented by slow centrifugation, and the supernatant fluid containing the living nucleated cells in uniform suspension is separated by aspiration.

*Technic of cell separation* Withdraw the desired amount of blood or marrow by venipuncture or sternal puncture and deposit in tubes with a measured amount of an anticoagulant. Sodium citrate, potassium oxalate or heparin are satisfactory for this purpose. Add the optimal amount of bean extract containing the phytohemagglutinin and mix. Centrifuge at about 40 g (500 rpm in an International centrifuge size 1, type S B, with No. 20354 head) for about 90 seconds or until the maximum plasma volume containing a uniform suspension of leukocytes with no buffy layer is obtained. Aspirate the supernatant plasma.

If the erythrocyte count is over 7,000,000 per cu. mm., add two volumes of pooled plasma and double the usual amount of bean extract, mix thoroughly and proceed as above. Physiologic saline is not satisfactory as a diluent for polycythemic bloods. This technic will increase the yield of nucleated cells and the percentage of erythrocytes eliminated in nonpolycythemic bloods, but is not necessary for most purposes.

*Preparation and standardization of the phytohemagglutinin* Soak 200 Gm. of dry red beans (*Phaseolus vulgaris*) or navy beans (*Phaseolus communis*) for 24 hours in 1000 ml. of 0.85 per cent sodium chloride solution at room temperature. Macerate in a Waring blender. Let the mixture stand at room temperature for three hours, stirring frequently. Centrifuge and decant the viscous extract from the bean pulp and mix it with about 10 Gm. of kieselguhr or other filter-aid. Filter the extract through Whatman No. 30 paper in a Buchner funnel with suction. This may require

From the Division of Experimental Medicine, University of Oregon Medical School, Portland, Oregon, aided by a grant from the Medical Research Foundation of Oregon, Portland, Oregon.

16 to 24 hours because of the viscosity of the extract. Adjust the pH of the filtrate, usually about 4.2, to 7.3-7.6 with 20 per cent sodium hydroxide, add filter-aid and refilter through Whatman No. 30 paper in a Buchner funnel with suction. Sterilize this filtrate by passing through a Seitz filter. The yield is approximately 300 ml.

An amorphous material of high agglutinating titer may be obtained by dialysis and evaporation of the clear fluid after removal of the precipitated globulins as described by Osborne, Mendel and Harris<sup>1</sup> if the minimal possible organic addition is desired. However, the crude extract is entirely satisfactory for the separation of nucleated blood and marrow cells and is quite stable. Two aliquots of the extract showed no appreciable loss of potency after being kept for six months at room temperature (18 to 22 C) and for nine months at refrigerator temperature (8 to 10 C).

*Titration of the extract.* Do a leukocyte count on a sample of oxalated normal human blood and calculate the number of leukocytes in each 5 ml. Add to a series of centrifuge tubes, each containing 5 ml of this blood, increments of 0.05 ml (0.05 ml, 0.10 ml, 0.15 ml, etc.) up to 0.3 ml of bean extract. Mix thoroughly and centrifuge at 500 rpm (40 g) for 90 seconds. Enumerate the leukocytes in the supernatant plasma. From the volume of the supernatant plasma and its cell count calculate the yield of leukocytes as per cent of the absolute number in the original 5 ml of whole blood. The volume of bean extract which produces the highest yield of leukocytes and the fewest erythrocytes is the optimal amount for that lot of extract. This is usually 0.1 to 0.2 ml of the extract for each 5 ml of oxalated blood. Too little extract fails to agglutinate all the erythrocytes and too much entraps some leukocytes in the large aggregates of erythrocytes produced.

*Efficiency of the separation.* Tables 1, 2 and 3 show the data obtained in evaluating the method. As shown in table 1 the phytohemagglutinin affects the erythrocytes of all blood groups equally efficiently. In bloods with essentially normal leukocyte and erythrocyte counts, a mean of 77 per cent of the total leukocytes present in the original blood were recovered in the plasma, with a range of 61 to 94 per cent. A mean of 99.82 per cent of the erythrocytes originally present were eliminated, with a range of 99.5 to 99.98 per cent. The percentage recovery shows no relationship to the initial leukocyte or erythrocyte counts.

Note from table 2 that 52 to 78 per cent of the leukocytes of leukemic bloods with high cell counts were recovered and over 99 per cent of the erythrocytes present in the original blood were eliminated. There appear to be no significant differences in the recovery of leukocytes from the different types of leukemia. It is shown in table 3 that when the technic recommended for polycythemic bloods was employed 60 to 100 per cent of the leukocytes were recovered and over 99 per cent of the erythrocytes were eliminated.

Cell separations carried out by this technic on marrows from patients with marked hyperplasia of the erythrocytic series of cells have shown comparable recoveries of the total nucleated erythrocytic cells with proportions of each nucleated erythrocytic stage similar to those in the original marrow as shown by differential counts of smears prepared before and after cell separation. It is obvious,



TABLE 1.—Percentage of Leukocytes Recovered and of Erythrocytes Eliminated in Separations on Bloods of Medical Students and Laboratory Personnel

Blood Group	WBC per cmm. Blood	% Yield of WBC*	RBC per cmm Blood	% RBC Eliminated†
O	10,200	66.6	5.2 ml.	99.98
O	7,600	82.6	4.5	99.83
O	7,400	82.6	4.9	99.87
A	9,350	80.2	5.5	99.86
A	13,700	79.6	5.1	99.98
A	10,000	73.5	5.5	99.92
A	7,400	68.2	4.7	99.76
B	6,500	77.4	5.2	99.98
B	10,900	83.7	5.0	99.37
B	6,600	65.5	5.2	99.95
B	8,900	61.4	5.7	99.87
AB	12,300	94.0	4.8	99.53
AB	5,600	82.5	3.7	99.75
Mean		76.8		99.82

\* WBC per cmm. of separated plasma  $\times$  volume of plasma  
WBC per cmm of whole blood  $\times$  volume of blood  $\times 100 =$  % Yield of WBC.

†  $100 - \left( \frac{\text{RBC per cmm of separated plasma} \times \text{volume of separated plasma} \times 100}{\text{RBC per cmm of whole blood} \times \text{volume of whole blood}} \right) =$  % RBC eliminated.

TABLE 2.—Percentage of Leukocytes Recovered and of Erythrocytes Eliminated in Separations on Leukemic Bloods

Blood Group	WBC per cmm. Blood	% Yield of WBC	RBC per cmm Blood	% RBC Eliminated
A*	234,500	71.6	4.5 ml	99.97
A*	298,000	60.0	4.5	99.30
A*	27,000	59.4	3.4	99.51
B*	146,400	69.6	3.9	99.50
AB*	230,000	52.4	4.2	99.91
O†	310,500	77.8	2.2	99.69
A†	90,000	57.5	3.5	99.27
B†	262,200	59.4	2.1	99.35

\* Chronic granulocytic leukemia.

† Chronic lymphocytic leukemia.

‡ Acute monocytic leukemia

TABLE 3.—Percentage of Leukocytes Recovered and of Erythrocytes Eliminated in Separations on Polycythemic Bloods

Blood Group	WBC per cmm Blood	% Yield of WBC	RBC per cmm Blood	% RBC eliminated
O	31,000	60.0	8.5 ml.	99.50
A	11,000	72.0	10.5	99.97
A	13,500	77.2	8.2	99.95
A	13,500	100.0±	8.2	99.80
A	8,000	82.5	9.8	99.41
B	7,150	87.4	7.7	99.64
B	26,000	62.4	9.0	98.93
AB	21,000	93.6	10.7	99.10

therefore, that the phytohemagglutinin does not agglutinate nucleated erythrocytes in appreciable numbers even when they have developed their full complement of hemoglobin. Reticulocyte counts from the supernatant fluid indicate that the proportions of reticulocytes in the few remaining erythrocytes are somewhat increased, but that the majority of reticulocytes are eliminated.

### DISCUSSION

*Properties of the phytohemagglutinin* Although we rediscovered this phytohemagglutinin accidentally and independently, search of the literature revealed that Dorset and Henley<sup>2</sup> in 1916 used a crude extract of navy beans (*Phaseolus communis*) in the large scale preparation of antiserum for hog cholera. Goddard and Mendel<sup>3</sup> in 1929 thoroughly investigated the properties of the phytohemagglutinin isolated from navy beans, described a technic for preparing it in purified form and showed that it agglutinated human, rabbit, dog, mouse, chicken, duck and rat erythrocytes. It differs from concanavallin-A<sup>4</sup> in that it agglutinates human erythrocytes and from ricin<sup>1</sup> in that it is nontoxic. We have cultured leukocytes and nucleated erythrocytes isolated by this technic, using the marrow culture method,<sup>5, 6</sup> and have noted no detectable effects on the morphology, survival, mitosis or motility of the cells. Goddard and Mendel<sup>3</sup> injected 8 mg. of their purified material per kilogram of body weight into rabbits and 600 mg. per kilogram of body weight into mice with no demonstrable deleterious effects. This lack of toxicity clearly shows that it is not ricin, which is one of the most toxic substances known. Goddard and Mendel<sup>3</sup> concluded from their studies of the purified material that it is a water-soluble albumin. They found that 0.7 micrograms of the purified material would completely agglutinate 0.5 ml. of a 2.5 per cent suspension of human erythrocytes in isotonic saline. We found that 0.1 mg. of the purified material was optimum for agglutination of the erythrocytes in 1 ml. of normal blood. Use of the purified material is necessary only if the minimal amount of contamination by extraneous organic material is essential in the studies for which the isolated leukocytes are to be used.

*Comparison with other methods* The usual method of separation of leukocytes and nucleated erythrocytes by prolonged centrifugation to concentrate them in a layer has several disadvantages. Among these are the contamination of the leukocytes by erythrocytes, the low yield of 30 to 50 per cent and the clumping which prevents accurate cell counts.

A number of methods have been reported recently designed to overcome these disadvantages. None of these, however, introduces so little extraneous organic material, gives such consistently satisfactory cell separations and leaves the nucleated cells in both a countable and viable condition. In none of the other reports are adequate data given from which to calculate the percentage yields of nucleated cells. None of these reports describes the separation of nucleated erythrocytes.

Of these methods the technic described by Valec, Hughes and Gibson<sup>7</sup> of flotation of leukocytes on salt-free albumin of specific gravity 1.079 appears to be the best. This method is not suitable for processing large volumes of blood. The adjustment of specific gravity of the albumin solution has to be very critical and somewhat different for different blood or marrow specimens. Cells at the albumin-

plasma interface are exposed to high colloid osmotic pressures and may be markedly shrunk and the cells are no longer countable because of matting together. One can never be certain what the yield is on the particular blood or marrow specimen under study. We have found difficulty with this method in getting uniform, complete separation of the nucleated and non-nucleated cells, although under ideal conditions it probably gives the highest percentage yield of any method yet described.

The method of Spear<sup>8</sup> uses the same flotation principle and has the same advantages and disadvantages as the preceding method plus the necessity of freeing the cells from the gum acacia used and the technical difficulty of adjusting the solution to proper pH, tonicity and specific gravity. It is much more time-consuming than the method here presented.

The method of Singer, Silberbach and Schwartz<sup>9</sup> depends on hemolysis of the erythrocytes by a mixture of gramicidin and lysolecithin. Free hemoglobin is known to be toxic to cultures of living cells. Intensive washing of a highly viscous mixture is necessary to free the nucleated cells from hemoglobin. It seems highly probable that these surface active substances might damage the cytoplasmic membranes of leukocytes as well as erythrocytes.

The method of Minor and Burnett,<sup>10</sup> which depends on production of rouleau formation by addition of fibrinogen from fraction I of human plasma, appeared after this study was completed. While no studies of the yield obtained were reported, our work with the method indicates that if modified by the use of the centrifugation technic herein outlined satisfactory cell separation is obtained. It would be much more expensive if the fraction I were sold at cost of production. It also introduces larger amounts of extraneous organic and inorganic materials than the method described in this paper.

#### SUMMARY

A method is described for rapid and efficient separation of leukocytes and nucleated erythrocytes from blood or marrow. It is based on the rediscovery of a non-toxic hemagglutinin, isolated from common red or navy beans, which agglutinates all human erythrocytes and those of the animals which have been tested with it. The time from drawing the blood to complete separation of the cells is less than ten minutes. The cells remain in suspension in their own plasma and are countable. Negligible amounts of foreign material are introduced, a great advantage in chemical studies. The cells so isolated are living and suitable for culture studies. Any volume of blood from less than 1 ml. to over 500 ml. may be processed. In marrow nucleated erythrocytes are separated with the leukocytes and in their original proportions. The volume, motility, morphology and life span of the cells in cultures are not altered by addition of the bean extract.

#### REFERENCES

- <sup>1</sup> OSBORNE, T. B., MENDEL, L. B. AND HARRIS, I. F. The study of the proteins of the castor bean with special reference to the isolation of ricin. *Am. J. Physiol.* 14: 259, 1905.
- <sup>2</sup> DORSET, M. AND HENLEY, R. R. Production of clear and sterilized anti-hog-cholera serum. *J. Agr. Research* 6: 333, 1916.

- <sup>3</sup> GODDARD, V. R., AND MENDEL, L. B. Plant hemagglutinins with special reference to a preparation from the navy bean. *J. Biol. Chem.* 82: 447, 1929.
- <sup>4</sup> DAMESHEK, W., AND MILLER, E. B. Pathogenetic mechanisms in hemolytic anemias. *Arch. Int. Med.* 72: 1, 1943.
- <sup>5</sup> OSGOOD, E. E. Marrow culture, *A Symposium on the Blood*, pp. 219. Madison: Wisconsin University of Wisconsin Press, 1941.
- <sup>6</sup> — Culture of human marrow. Summary of studies to date. *West. J. Surg., Obs. and Gyn.* 48: 540, 1940.
- <sup>7</sup> VALEE, B. L., HUGHES, W. L., AND GIBSON, J. G. A method for the separation of leukocytes from the whole blood by flotation on serum albumin. *Blood*, Special Issue No. 1, 1947.
- <sup>8</sup> SPEAR, F. Separation of leukocytes from whole blood by flotation on gum acacia. *Blood* 3: 1055, 1948.
- <sup>9</sup> SINOER, T. P., SILBERBACH, I., AND SCHWARTZ, S. The preparation of morphologically intact leukocytes from peripheral blood by means of gramicidin and lysolecithin. *Blood*, Special Issue No. 1, 1947.
- <sup>10</sup> MINOR, A. H., AND BURNETT, L. A method for obtaining living leukocytes from human peripheral blood by acceleration of erythrocyte sedimentation. *Blood* 3: 799, 1948.

# ABSTRACTS

JOSEPH F ROSS M.D. *Editor*

## ABSTRACTERS

HELEN W BELDING M.D. Winstan Salem N C	OLIVER P JONES Ph.D. Buffalo
SHEILA T CALLENDER M.D. Oxford England	CONRAD MAIER, M.D. Zurich Switzerland
GEORGE E CARTWRIGHT M.D. Salt Lake City	MILOŠ NETOUŠEK, M.D. Prague Czechoslovakia
ROGER C CRAFTS Ph.D. Boston	JEAN P SOULIER, M.D. Paris
CHARLES P EMERSON M.D. Boston	RAMON M. SUÁREZ, San Juan Puerto Rico
SOLOMON ESTREN M.D. New York	WILLIAM VALENTINE, M.D. Los Angeles
CLEMENT A FINCH M.D., Boston	JAN WALDENTRÖM M.D. Upsala Sweden

## HEMOLYTIC ANEMIAS

HAEMOLYTIC DISEASE OF THE NEWBORN CRITERIA OF SEVERITY P. L. Mellison and Marie Catbush From the Medical Research Council Blood Transfusion Research Unit Department of Obstetrics Post graduate Medical School, London Brit M J 1 123 1949

The hemoglobin value of cord blood is well correlated with the severity of hemolytic disease. In contrast venous and capillary blood samples especially when taken some hours or days after birth are liable to misinterpretation. Normal values do not exclude anemia at birth. If the cord is not clamped early and transfer of placental blood is allowed, a deceptively high hemoglobin value may result.

In this series most of the babies with a cord hemoglobin of less than 8 grams per cent died within twenty four hours of birth. A raised venous pressure found in some of these infants suggested that they died from heart failure. All of those with a cord hemoglobin of over 14 grams per cent survived.

The cord bilirubin and degree of erythroblastemia also show some correlation with severity of the disease but other tests such as the strength of the direct Coombs test amount of free antibody in the infants blood and form of antibody in the mothers serum are of limited value.

These observations are of considerable value as a guide to the management of infants with hemolytic disease. Also, as the authors point out the adoption of examination of cord blood as a routine in affected babies would make it possible to compare groups of cases in regard to severity.

S.C.

MEDITERRANEAN ANEMIA A. Marmont and V. Bianchi From the University Medical Clinic Geneva (Italy) Acta haematologica 1 4-18 1948

Three cases in the same Sicilian family of the so-called Rietti-Greppi Micheli's hemolytic anemia with increased osmotic resistance of the erythrocytes are reported remarkable also for the clinically and hematologically normal parentage. The genetic implications related to this point are discussed. The findings of a biliary calculus and an ulcer on the right ankle of one of them are briefly elucidated. Hematologic studies showed the well known features of this peculiar disease particular stress is laid on the authors' researches on red cells fragmentation *in vitro* and *in vivo* chiefly by means of a supravital staining technique and others.

The demonstration of an intense erythrocytic disintegration with signs of increased mechanical fragility, both in this and in genuine Cooley's anemia affords still a further motive to the authors for identifying the above mentioned syndrome with the moderate form of Mediterranean anemia. The hypothesis of mechanical fragility as closely related to increased osmotic resistance and inversely to osmotic fragility is considered and an outline of a new classification of primarily hemolytic and primarily erythrocytic anemias the latter comprising both thalassemia and sickle-cell anemia is put forward. Some

speculations as to the nature of the blood disorder suggesting a congenital error of iron metabolism possibly involving faulty stromato-hemoglobin bindings, are advanced

C M

**CONGENITAL HEMOLYTIC ICTERUS IN THE NEGRO** R. R. McCormack and E. P. Simon From the Department of Medicine, Cornell University Medical College and the Second (Cornell) Medical Division Bellevue Hospital New York City *Am J M Sc* 216 539-544 1948

Congenital hemolytic icterus in a 16 year old Negress is reported. This patient is a maternal aunt of the patient reported by Scherer and Cecil (*J Lab & Clin Med* 30 244 1945) and if the diagnosis is correct is about the fourth or fifth well authenticated case reported in a Negro. No evidence of racial admixture was obtained. The diagnosis of congenital hemolytic icterus was supported by the family history, spherocytosis, splenomegaly, and increased erythrocyte fragility in hypotonic saline. However history of hemolytic episodes was singularly lacking, the anemia and reticulocytosis were minimal and although jaundice was present it was most likely due to the passage of a common duct stone. Following splenectomy and cholecystectomy the jaundice disappeared, the spherocytes diminished in numbers the saline fragility curve shifted toward normal and the reticulocytosis, macrocytosis and anemia disappeared.

G E C

**THE SICKLE CELL TRAIT: INCIDENCE AND INFLUENCE IN PREGNANT COLORED WOMEN** P. K. Switzer and H. H. Fowble From the Departments of Medicine and of Obstetrics and Gynecology of the Medical College of the State of South Carolina and of the Roper Hospital *Am J M Sc* 216 330-332, 1948

The sickle cell trait was found in 14.2 per cent of 500 gravid Negro women, in 14 per cent of 250 nongravid Negro females of child bearing ages, and in 13.8 per cent of 250 adult Negro males. Of 71 pregnant females with the sickle cell trait, one was found to have mild sickle cell anemia. Sickle cell anemia did not interfere with conception nor with normal pregnancy and delivery. Pregnancy did not activate a blood destructive process in 21 sickle cell patients observed through the last trimester and during labor and the puerperium.

G E C

**HAFS DISEASE IN SWEDEN** R. Berlin From the Medical Department of the Lidköping County Hospital Lidköping, Sweden *Acta med Scandinav* 129 560 1948

The disease observed by Berlin in 11 cases from Central Sweden has previously never been observed outside East Prussia. It was first described in 1924-25 and later in 1932-33 with in all about 1000 cases. Even if the disease itself is hardly to be regarded as belonging to hematology the sudden appearance of large amounts of blood pigments in the urine should make it of diagnostic importance especially to hematologists.

The symptoms appear some time after the patient has eaten fish and occur chiefly among fishermen. In the present epidemic all the patients lived around a lake where the fishing is of great economic and nutritional importance. All the cases occurred from February 1942 to April 1943. Later there were no similar cases. The symptoms are always ushered in by pains in the legs and back, the urine is dark red and contains large amounts of myoglobin. After some days the pains disappear and the urine becomes pale. Uremia is the only complication and in the author's material there were 2 deaths among 11 persons.

The author discusses the possibility that the disease may have something to do with the so-called Chastek paralysis caused by the presence of an antithiamin active substance in the muscles from certain fish species.

It seems probable that this diagnosis will be found to be more common with increasing knowledge of the symptomatology.

J W

**DIFFERENTIAL DIAGNOSIS OF HEMOLYTIC ANEMIAS** H. Ludin From the Medical University Clinic Basel (Switzerland) *Acta haematologica* 1 28-33 1948

The paper reports on the determination of the life duration of transfused red cells in two cases of hemolytic anemia, one of these cases belonged to macrocytic anemia, Dyke Young, the other was a case

of congenital hemolytic jaundice. The conclusions which can be drawn from these investigations for the pathogenesis of such types of anemia are discussed

C.M.

A CASE OF LYMPHOGRANULOMATOSIS (HODGKIN'S DISEASE) WITH HEMOLYTIC ANEMIA *Svend Grønlund*  
From the Medical Department of Aalborg County Hospital, Denmark. *Acta med Scandinav* 129: 361, 1947

The occurrence of hemolytic anemia (hypersplenism) in patients with splenomegaly from different causes is well known at present. The case treated in this paper is of great interest as splenectomy caused temporary improvement of the anemia (erythrocytes from 0.84 mill to 3.5 mill). Later the patient developed typical Hodgkin's disease with involvement of the lymph glands. Histologic structure was typical in the glands but the microscopic examination of the spleen showed no sign of Hodgkin's disease. Two similar cases have been published previously. Such instances of symptomatic hypersplenism are of great therapeutic importance as has recently been emphasized by Dameshek.

J.W.

## MACROCYTIC ANEMIAS

MACROCYTIC ANEMIA IN CENTRAL AFRICANS IN RELATION TO ANCYCLOSTOMIASIS AND OTHER DISEASES. *H. Lehmann*. From Makerere College and Mulago Hospital, Kampala, Uganda. *Africa Lancet* 1: 90-95, 1949

This article contains some interesting observations which help to disentangle some of the problems of both tropical macrocytic anemias and Kwashiorkor.

By the study of 44 cases of severe anemia, the author shows that macrocytosis in the Central African is due mainly to reticulocytosis in response to blood loss or following appropriate treatment. These macrocytes he calls coctics (unfinished) cells being distinct from macrocytes derived from megaloblasts. Most of the patients had a severe hypochromic type of anemia due to hookworms but malaria and infection in some masked the blood picture of iron deficiency. Evidence is given that either iron or worming will give a partial remission in hookworm anemia but that both are necessary for full recovery. It was further noted that worming reversed symptoms usually associated with Kwashiorkor, e.g., pale skin and hair, in patients whose iron deficiency has been corrected. The suggestion is made that the parasites inhibit tyrosine oxidation, thus affecting melanin formation and arresting maturation of reticulocytes. This might be an additional factor in producing macrocytes. Tests in which tyrosine was injected into the skin are described to support the idea of inhibition of oxidation by parasites.

S.C.

A CASE OF FERNICIOUS FORM ANEMIA IN A CHILD NINETEEN MONTHS OLD. *P. H. D. Waagstein*. From the Medical Service of the County Hospital, Maribo, Denmark. *Acta med Scandinav* 131: 347, 1948

The child had been breast fed until 10 months old. Then for four months chiefly breast fed. Later milk but practically no egg, fish or meat. At 19 months, severe megalocytic anemia with megaloblastic marrow was found. Gastric acidity over 40 units of free HCl after histamine. Treatment with concentrated liver extract gave reticulocyte response and prompt changes in the sternal marrow. After thirty days the red cell count was 4 million. Specific treatment was stopped and after six months there was a severe relapse. Liver extracts had excellent effect again. The diet in the interval was regarded as sufficient.

J.W.

A CASE OF REFRACTORY ANEMIA IN A FINAL STATE SUGGESTIVE OF APLASTIC ANEMIA WITH INCREASED PIGMENTATION OF THE SKIN SUCCESSFULLY TREATED WITH FOLIC ACID. *B. Andersson*. From the Medical Department of the Caroline Hospital in Stockholm. *Acta med Scandinav* 130: 468, 1948

A case of chronic liver refractory macrocytic anemia with nonmegaloblastic marrow and no increase in reticulocytes, no signs of liver damage and presence of free HCl in the gastric juice was treated with blood transfusions until folic acid could be given. With 25 mg. of this preparation *pro die* there was rapid improvement with marked reticulocytosis and increase in both red and white cells. Such cases are of great importance as folic acid seems to be the only way of treating them effectively. The presence of free

HCl in the gastric contents in such atypical liver refractory conditions that respond well to the administration of folic acid should be especially stressed (cf also macrocytic anemia of pregnancy)

J W

ON THE PRICE JONES CURVE IN INITIAL PERNICIOUS ANEMIA *G Tetterman* Helsingfors Acta med Scandinav 129 478 1948

Thirteen patients with initial pernicious anemia were investigated with regard to skewness of the Price Jones curves. The majority of the cases had symmetric curves but the distribution range of the cell-sizes was abnormal showing the blood to be pathologic

J W

PORPHYRIN IN PERNICIOUS ANEMIA *C D De Langen* Medical University Clinic, Utrecht (Holland) Acta haematologica 1 93-98 1948

Liver extracts contain a substance that can combine with porphyrin. The porphyrinic properties are lost as long as it forms part of this compound. This hitherto unknown substance is found especially in the liquid obtained by expression of the liver. In pernicious anemia it is lacking. In urine and stomach secretion of normal persons this substance is always present but in urine and stomach of patients with pernicious anemia it was not found even after treating these patients with liver extracts

C M

FOLIC ACID IN THE TREATMENT OF PERNICIOUS TAPEWORM ANEMIA *B von Bonsdorff* Helsingfors Finland Acta med Scandinav Suppl 213, 81-90 1948 Studia in Honorem Einar Meulengracht

This paper is a continuation of Bonsdorff's previous work on the mechanism of tapeworm anemia in Finland. Folic acid had an excellent effect in four cases of this disease when given in doses of 20-30 mg perorally for 7-10 days. The author concludes that the antianemic effect is not impaired by the presence of the worm in the intestinal canal

J W

DOES FEEDING OF DIPHYLLOBOOTHRIUM LATUM INFLUENCE THE INTERACTION BETWEEN THE INTRINSIC AND THE EXTRINSIC FACTORS OF CASTLE? *B von Bonsdorff* Helsingfors Acta med Scandinav 129 59 1947

The possibility that the tapeworm might contain some substance antagonistic to the action of the antianemic factor formed by the interaction of Castle's intrinsic and extrinsic factors was tested by the author. Neither fresh nor dried tapeworm has any influence on this interaction in vivo. Nor was the remission after the expulsion of the worm checked by peroral administration of dried tapeworm. Preparations of hog's stomach mixed with large amounts of dried worm had retained their therapeutic effect

J W

IN WHICH PART OF THE INTESTINAL CANAL IS THE FISH TAPEWORM FOUND? *B von Bonsdorff* Helsingfors Finland Acta med Scandinav 129 142 1947

The explanation of the fact that only a low percentage of tapeworm carriers show signs of pernicious anemia is not yet found. The possibility that the location of the worm may be of importance from the point of view of pernicious anemia was investigated. It was found that the worm is most frequently located in the ileum rarely in the jejunum and very rarely in the gall bladder. Vomiting of the tapeworm seems to be connected with a higher incidence of anemia. The possibility that the tapeworm may be located higher up in the intestinal canal when vomited seems worth discussing. The author is very careful however in drawing any conclusions regarding the connection between high location of the worm and occurrence of tapeworm anemia

J W

ON THE SECRETION OF GASTRIC JUICE IN RECOVERY AFTER PERNICIOUS BOTHRIOCEPHALUS ANEMIA *C A Hernberg* From the Medical Department of Maria Hospital Helsingfors Acta med Scandinav 129 12, 1947

A follow up examination of 24 patients who had suffered from pernicious tapeworm anemia 1-22 years ago is published. The blood picture was normal. Achlorhydria was found in 11 cases. In all of these



the worm had been expelled only 2-3 years ago. In the other cases 7-9 years had elapsed since the expulsion of the worm. No case was found where an idiopathic pernicious anemia had developed.

J W

## ANEMIA THERAPEUTIC AGENTS

THYMIDINE AND VITAMIN B<sub>12</sub> IN PERNICIOUS ANEMIA. C C Ungley. From the Royal Victoria Infirmary, Newcastle upon Tyne, England. *Lancet* 1: 164-165, 1949.

Thymidine has been isolated from liver and found to prevent the toxicity of methyl folic acid. It can also replace vitamin B<sub>12</sub> in the nutrition of certain lactobacilli. Such microbiologic evidence suggested that thymidine might have antipernicious anemia activity.

This paper reports that 48 mg. of thymidine intramuscularly produced no hematologic remission in a patient with classic Addisonian pernicious anemia. The same patient later responded to 7.5 µg. of a red crystalline antipernicious anemia factor identical with or closely allied to vitamin B<sub>12</sub>.

S C.

THE MAINTENANCE OF PATIENTS WITH TROPICAL SPRUE BY MEANS OF MASSIVE DOSES OF SYNTHETIC 5 METHYL URACIL (THYMINE). G G Lopez, F Milanes, R L Toca, T Aramburo and T D Spies. From the Department of Nutrition and Metabolism, Northwestern University and the Department of General Pathology, University of Havana, Havana, Cuba. *Am J M Sc* 216: 270-274, 1948.

Three patients with tropical sprue have been maintained in complete hematologic remission for at least a year on oral thymine therapy. There was no evidence of subacute combined degeneration at any time. The patients, according to the authors, were asymptomatic with completely normal stools after the first six weeks of therapy. Two of the patients were maintained on 5 grams of thymine per day. The third patient received 15 grams of thymine for 30 days and none thereafter.

G.E.C.

EFFECT OF PTERIDINES AND BLOOD SERA ON HUMAN BONE MARROW CELLS IN VITRO. E R Norris and J J Majnarić. From the Department of Biochemistry, University of Washington, Seattle, Washington. *Am J Physiol* 153: 496-498, 1948.

Pteridines and blood sera have been shown to cause a cellular proliferation in vitro of bone marrow suspensions of the rat, rabbit, cat, sheep, and beef. This work was done to determine the effects on the bone marrow of the human. Rib marrow was obtained by surgery and a suspension was prepared in Tyrode's solution without glucose. Ten mg. of hydrolysate and 0.5 mg. of tryptophane were added per ml. of cell suspension. Proliferation was determined by means of cell counts. Xanthopterin increased the rate of cell proliferation while antixanthopterin inhibited this proliferation.

R.C.C.

A METHOD FOR STUDYING THE EFFECT OF VARIOUS SUBSTANCES UPON RED CELL MATURATION IN VIVO. E E Hays. From the University of Vermont College of Medicine, Burlington, Vermont. *Am J M Sc* 216: 528-533, 1948.

An in vitro cell survival technic using rat bone marrow and based on the reticulocyte increase after three hours incubation at 38°C in various dilutions of glucose free Tyrode's solution is described. Data are given which indicate that maturation of the red cells is stimulated by anti-pernicious anemia liver extracts and not by various other substances including an inactive liver extract. Pteroylglutamic acid and pteroylheptaglutamic acid were inactive when so assayed. Normal human serum and rat serum exhibited the presence of the maturation factor required by this technic.

G.E.C.

INFLUENCE OF THE DOSAGE OF XANTHOPTERIN UPON THE RESPONSE IN HEMOPOIESIS. E R Norris and J J Majnarić. From the Department of Biochemistry, University of Washington, Seattle, Washington. *Am J Physiol* 153: 133-137, 1948.

Young rats were made anemic by feeding a purified diet containing one per cent sulfathiazole. A single injection of less than 5 mg. of xanthopterin per kilogram of body weight produced hemoipoiesis. At

results were obtained with 10 mg. Doses of 20 mg. or more aggravated the anemia. Normal rats were also studied. Similar results were obtained.

RCC

**INTRAVENOUS IRON THERAPY** *A. J. Agner, N. S. E. Andersson and N. G. Nordinson* Mediz. Klinik und Chem. Laboratorium des Serafimerlasarettet & II. Med. Poliklinik, Stockholm. *Acta haematologica* 1: 193-211, 1948.

Twenty cases of iron-deficiency anemias were treated with intravenous injections of a special chemical compound of ferric iron. In 17 cases an increase of the hemoglobin, the number of the erythrocytes, the reticulocytes and the serum iron was observed as an effect of this treatment. In 19 cases there was a special favorable effect on the symptoms and on the epithelial signs.

Side effects in connection with the injections were not noticed. Paravenous injection of the iron solution must be avoided.

CM

**INTRAVENOUS IRON IN THE TREATMENT OF ANAEMIA OF PREGNANCY** *A. D. T. Gowan and J. M. Scott* From the Glasgow Royal Maternity and Women's Hospital, Scotland. *Lancet* 1: 14-16, 1949.

Ferrivenin (saccharated oxide of iron) was used to treat 25 patients with anemia of pregnancy. All the patients responded to treatment and the response compared favorably to that obtained in 62 similar patients given iron by mouth. Two patients apparently refractory to iron by mouth responded to the intravenous iron. One patient had a severe reaction and about 10 per cent showed a slight general reaction at the first or second injection but not subsequently.

The pregnant anemic women appeared to need more iron than nonpregnant women to restore the hemoglobin values to normal, i.e. almost 40 mg. of iron for every 1 per cent increase in hemoglobin, perhaps due to the demands of the fetus.

SC

**INTRAVENOUS TREATMENT OF ANAEMIA WITH AN IRON-SUCROSE PREPARATION** *H. G. B. Slack and J. F. Wilkinson* From the Department of Haematology, Manchester Royal Infirmary, England. *Lancet* 1: 11-14, 1949.

After trial of many different iron preparations it was found that Seitz filtered ferric oxysaccharate B.P. (1 per cent iron) could be given intravenously in large doses without producing toxic symptoms. Sixty patients with iron deficiency anemia were treated: about a third of them with a home-made preparation and the remainder with Ferrivenin (Bengers Ltd.). The total blood iron deficit was calculated in each patient and 50 per cent added for depleted body stores. The usual scheme of dosage was 25 mg., increasing daily up to 200 mg. on the fourth and subsequent days until the total calculated dose had been given. Only one patient developed a mild reaction to 200 mg. but larger doses gave more frequent reactions. Fifty-seven of the 60 patients, including some refractory to oral iron, showed a striking therapeutic response. Utilization of iron appeared to be nearly 100 per cent, urinary loss being negligible. Two patients with chronic infection showed a response but required more than the calculated amount to maintain improvement.

This experience with intravenous saccharated oxide of iron confirms a real therapeutic advance. The suggestion that the anemia of infection may respond to this form of iron is especially interesting and clearly needs confirmation.

An amendment to the method of preparation and further details are given in a letter from these authors in *Lancet* 1: 163, 1949. They also give a warning against the irritant action of the iron preparation if allowed to leak around the vein.

SC

## LEUKOCYTES, LEUKEMIA, LYMPHOMA

**THE DIFFERENTIATION OF THE NEUTROPHIL LEUKOCYTES** *K. Reber* Mediz. Universitätsklinik, Zürich. *Acta haematologica* 1: 98-108, 1948.

The conception of the neutrophil stab-cell is critically analyzed and it is shown that by strictly using Schilling's definition only the lack of segmentation of this cell group is taken into consideration. While

the name of stab-cells should express at the same time a lack of lobulation of the nucleus. It is pointed out, that the tendency to lobulation of the nucleus and the degree of segmentation of the nucleus have from the genetic point of view a different origin. The lobulation of the nucleus respectively the definitive number of the segments is dependent on the form of the maturing myelocytes but the extent of segmentation is regulated by the speed of maturation and emigration. Those two functions are controlled by the vegetative nervous system the formation of the cells is under parasympathetic the maturation and emigration under sympathetic influence. It is suggested that from now on one should distinguish only between segmented and unsegmented neutrophils and to record separately the number of the segments either developed or still in development.

C.M.

THE INFLUENCE OF PHYSICAL AND CHEMICAL AGENTS ON THE MOVEMENT OF LEUKOCYTES *P. Scharrer*  
Anatomisches Institut der Universität Zürich *Acta haematologica* 1 178-192, 1948

A method is described for the measurement of movements of isolated cells. The results of the measurement concern the influence of hypotony and hypertony, colchicine, acetone, alcohol, chloralhydrate, stilbestrol and cibazol and they are compared with the influence on the development of mitoses in fibrocytes.

C.M.

CHANGES IN THE BLOOD LEUCOCYTE LEVEL OF ADRENALECTOMIZED AND NORMAL RATS FOLLOWING ADMINISTRATION OF TYPHOID VACCINE *L. A. Lewis and I. H. Page* From the Research Division of the Cleveland Clinic Foundation, Cleveland, Ohio. *Am. J. Physiol.* 153 148-152, 1948

Previous work has indicated that toxic agents introduced into the body produce a decrease in the circulating lymphocytes through the action of the adrenal cortex. This work was done to determine whether there would be any response to typhoid vaccine in the absence of the adrenal glands. Fifty-six adult male rats of the Sprague Dawley strain were used. They were adrenalectomized and maintained on 0.9 per cent salt solutions. Intraperitoneal injections of typhoid vaccine markedly lowered the lymphocyte level two hours after the injection in the absence of the adrenal glands. In addition, the neutrophils increased in number. Normal human beings injected with typhoid vaccine showed similar results. It is concluded that the adrenal glands are not necessary for the lymphopenia which is induced by typhoid vaccine.

R.C.C.

THE NEGATIVE EFFECT OF FOLIC ACID ON IRRADIATION LEUKOPENIA IN THE CAT *W. S. Adams and J. S. Lawrence* From the University of Rochester School of Medicine and Dentistry and the Medical Department of Strong Memorial and Rochester Municipal Hospitals, Rochester, New York. *Am. J. M. Sc.* 216 656-660, 1948

The prophylactic and therapeutic administration of folic acid to cats did not alter the occurrence or the magnitude of leukopenia caused by exposure to 200 r whole body irradiation.

G.E.C.

STUDIES ON BLOOD HISTAMINE. PARTITION OF BLOOD HISTAMINE BEFORE AND AFTER CLOTTING IN HEALTH AND DISEASE STATES *W. N. Valentine and J. S. Lawrence* From the University of Rochester School of Medicine and Dentistry and the Department of Medicine of Strong Memorial and the Rochester Municipal Hospital, Rochester, New York. *Am. J. M. Sc.* 216 619-624, 1948

Data are presented on the partition of blood histamine before and after clotting and on the correlation of blood histamine levels with the blood leukocyte picture in health and disease states. The data support the view that most of the blood histamine in man is found in the myeloid leukocyte. Little or no transfer of histamine from cells to serum was observed when human blood was allowed to clot. Greatly increased values for blood histamine were found in patients with chronic myelogenous leukemia but no close correlation was obtained between the level of blood histamine and the total or differential leukocyte count. The studies did not permit any conclusions as to which members of the granulocyte series are richest in histamine content.

G.E.C.

LYMPHOCYTES AND INTRAVASCULAR HAEMOLYSIS *R. H. Trisick* From the South London Blood Supply Depot Sutton Surrey *Lancet* 1 225 1949

A patient with acquired hemolytic anemia accompanying Hodgkins disease showed a positive Coombs test. This was taken to indicate the intravascular hemolytic action of an immune gamma globulin. In stained films of the patient's blood many of the lymphocytes showed cytoplasmic buds especially at the time of maximum hemolysis and it is suggested that such buds becoming detached might be the source of the immune gamma globulin.

S.C.

ON SERUM COPPER IN ANGINA SIMPLEX AND IN INFECTIOUS MONONUCLEOSIS *S. March-Petersen* From the Biochemical Institute Aarhus University, from the Medical-epidemic Department, Aarhus Marselisborg Hospital and from the Medical Department of Aarhus County Hospital, Denmark *Acta med Scandinav* 131 588, 1948

Serum copper was determined with sodium diethyl-carbamate in 22 cases with angina and 20 patients with infectious mononucleosis. In both conditions the values were increased as might be supposed in a febrile condition. The values in infectious mononucleosis were much higher. The meaning of this difference is discussed but no explanations could be found.

J.W.

ARE THE BASOPHILIC LEUKOCYTES HEPARINOCYTES? *Guido Tøttersen* From the Second Medical Clinic, Helsingfors *Acta med Scandinav* 131 176, 1948

The author discusses the problem why cases of chronic myeloid leukemia with increase in the basophilic leukocytes do not show greater tendency toward bleeding than patients with the same disease but with low counts of basophilic cells. If the basophilic cells of the blood were really producing heparin this is hard to explain. The difference between tissue basophilic cells and blood basophilic cells is pointed out.

J.W.

ARTERIALGIC LEUKEMIA IN CHILDREN *J. Bichel* From the Clinical Department and the Research Laboratories of the Radium Centre of Jutland, University of Aarhus *Acta haematol* 1 153-164 1948

The author stresses the frequent occurrence of osteo-articular symptoms in the early stages of leukemia in children. Many such cases have for a time been mistaken for acute rheumatic fever. In early infancy rheumatic symptoms will always indicate a careful examination of the blood (often including the bone marrow as the blood picture may be almost normal until terminally). The relation between the articular symptoms and the radiographically demonstrable bone lesions is discussed. The value of the roentgenologic examination of the skeleton in obscure cases is emphasized. The literature is surveyed, and three characteristic case reports are presented.

C.M.

MONOCYTIC LEUKEMIA (CASE REPORT OF A NAEGLI TYPE) *H. Diekmann and H. van Ijzeren* From the Medical University Clinic, Utrecht (Holland) *Acta haematologica* 1 55-59 1948

Monocytic leukemia is briefly discussed and a case of the Naegli type is reported. The monocytoid elements which were originally found changed their character and more and more resembled myeloblasts, finally only paramyeloblasts and myeloblasts were observed. Sternal puncture made an early diagnosis possible.

C.M.

LYMPHOSARCOMA TERMINATING IN LYMPHATIC LEUKEMIA (LYMPHOSARCOMA CELL LEUKEMIA) *L. Hauswirth, G. Resnew and W. Lansman* From the Departments of Medicine and of Pathology, Beth David Hospital, New York New York *Acta haematologica* 1 45-54 1948

A case is presented in which the patient when first seen had the histologic findings of lymphosarcoma with normal blood and bone marrow a year later an acute lymphatic leukemia developed with rapidly fatal course. The transition of the lymphosarcomatous to the leukemic phase could be followed by serial blood, sternal and iliac bone marrow and tissue studies and confirmed at autopsy.

The cells of the blood marrow and the lymphnodes have the characteristic features of lymphosarcoma cells. Consequently the disease can be classified as lymphosarcoma cell leukemia.

The possibility is discussed whether ro-nitrogen and nitrogen mustard therapy induced or enhanced the appearance of the leukemic phase.

C M

THE ELECTROPHORETIC ANALYSIS OF SERUM PROTEINS OF THE BLOOD DYSCRASIAS. R. A. Brown, J. T. Read, B. A. Wiseman and W. G. France. From the Departments of Medicine and Chemistry, The Ohio State University, and the Starling Loving University Hospital, Columbus, Ohio. *J. Lab. & Clin. Med.* 33, 1523-1533, 1948.

The electrophoretic patterns of various blood dyscrasias are presented. The leukemic states are associated with a diminution in the approximate absolute amount of albumin and a rise in the absolute amount of globulin. The albumin globulin ratios fall below the limits of normal in most instances. The alpha 1 and alpha 2 globulin are increased in most instances and the increase is noted with both normal and diminished total albumin values. Gamma globulin values, both absolute and relative, were elevated in monocytic leukemia, reticulum cell sarcoma, and infectious mononucleosis. Chronic lymphatic leukemia demonstrated low relative and absolute gamma globulin values. A markedly lowered albumin globulin ratio appears related to the degree of infiltration of the bone marrow by leukemic cells as well as when the excretory and metabolic functions of the liver demonstrate impairment. No alteration in the serum protein architecture was noted following Stilbamidine therapy.

G.E.C.

THE INTERRELATIONSHIP OF HODGKIN'S DISEASE AND OTHER LYMPHATIC TUMORS. R. P. Carter and W. G. Bernhard. From the Army Institute of Pathology, Washington, D. C., and the Laboratories of the Presbyterian Hospital in Philadelphia, Pennsylvania. *Am. J. M. Sc.* 216, 625-642, 1948.

During the last war the authors were assigned to the Army Institute of Pathology to render a report on all lymphatic and hemopoietic tissue submitted. The present paper is a summary of a pathologic study involving 1300 lymphatic tumors including 700 cases of Hodgkin's material and 600 cases of lymphomas (follicular lymphoblastoma, lymphosarcoma, reticulum sarcoma, lymphatic leukemia, monocytic leukemia) apart from Hodgkin's disease. The authors conclude that a rigid subclassification of lymphatic tumors is artificial and confusing. In their material there was a striking fluidity in histologic pattern with transitions and combinations that could best be interpreted as indicating a single neoplastic entity having a number of variants. As they state, this is not surprising when one appreciates that all cellular components of lymphatic tissue are derived from the same mesenchymal stem cells.

A virtually complete alteration in the histologic pattern of the tumor in the Hodgkin's group was noted in 39 per cent of the 138 autopsied cases in which biopsies were available and in 31 per cent of the serial biopsy group. Pure tumor types were present in only 19 per cent of the autopsy group and in 23 per cent of the serial biopsy group. The variety of histologic appearances observed in different foci in the same individual, and even in several areas in the same node, was still more spectacular. Three hundred eighty-four of 700 cases presented these combined lesions. Lymphomas not grouped with Hodgkin's disease also exhibited an alteration of their histologic structure in much the same fashion.

G.E.C.

## BONE MARROW AND RETICULOENDOTHELIAL SYSTEM

THE OCCURRENCE OF EPITHELIOID CELL GRANULOMAS IN HUMAN BONE MARROW. H. Gormsen. From the University Institute of Legal Medicine, Copenhagen. *Acta med. Scandinav. Suppl.* 213, 154-164, 1948. *Studia in Honorem Einar Meulengracht*.

Histologic sections of sternal aspirates showed typical granulomas in 10 of 39 patients with Boeck's sarcoid, in 5 of 5 patients with milary tuberculosis, and in 15 of 22 patients with brucellosis.

J W

A HEMATOLOGICAL AND HISTOLOGICAL STUDY OF THE BONE MARROW AND PERIPHERAL BLOOD OF THE ADULT DOG. P. E. Rekers and M. Coulter. From the Department of Radiation Biology, The University of Rochester School of Medicine and Dentistry, Rochester, New York. *Am. J. M. Sc.* 216, 643-655, 1948.

This is probably the most extensive study of the peripheral blood and bone marrow of the normal dog which has as yet been published and has promise of serving as the standard reference of this subject for some time to come. It is regrettable, however, that volume of packed red cell measurements were not done and values for the red cell indices calculated. Analysis of the peripheral blood of 91 normal dogs is presented including differential white cell counts. Bone marrow differential counts from the ribs, femora, tibiae and humeri are presented, compared and analyzed statistically. Bone marrow total nucleated cell counts are given for one or more sites from 4 different bones. Rib bone marrow has been studied at 2, 3, 4, 5 and 8 hours after death and no significant alterations in degree of cellularity and cellular detail was found. Histologic studies are also included.

G E C

EXPERIMENTAL OBSERVATIONS ON THE STRUCTURE OF THE BONE MARROW. A. Nèzet. From the Université de Liège. Institut de Clinique et de Policlinique Médicales. Quart. J. Exper. Physiol. 34: 43-46, 1947.

This work was undertaken to determine whether ripe erythrocytes were stored in the bone marrow. Dog A was injected with 30 mg. of phenylhydrazine per Kg. of body weight which produces Heinz's granules in a large percentage of the dog's erythrocytes. After allowing time for all phenylhydrazine to be eliminated, the circulation of dog A is crossed with another dog, dog B. In this way the labelled erythrocytes were introduced into dog B. This crossed circulation was maintained from thirty minutes to one hour. By means of this technique the author found that no ripe erythrocytes were stored in the bone marrow and concluded that all red cells achieve their ripening in the circulating blood.

R C C

AN APPLICATION OF BONE MARROW CULTURES TO TOXICOLOGY AND THERAPEUTICS. K. Harrison and F. W. Randall. From the Chemical Defence Experimental Station, Porton, near Salisbury. Quart. J. Exper. Physiol. 34: 141-148, 1948.

The author points out that the intact animal is not always the best thing to use in the study of toxicology. He describes a method of using tissue cultures of bone marrow for the study of the toxic actions of drugs.

R C C

ARE NON NUCLEATED ERYTHROCYTES FORMED BY BUDDING OFF OF CYTOPLASM FROM NORMOBLASTS? Lissa Bæstrom. From Central Clinical Laboratory of Södersjukhuset, Stockholm. Acta med. Scandinav. 131: 303, 1948.

The still unsolved problem of the denucleation of red blood cells or the separation of cytoplasm from the erythroblasts in the marrow is discussed. A large number of morphologic observations that seem to indicate that the hypothesis of protoplasmic budding may be correct are presented. Also the bone marrow cultures by Plum are regarded as proof that this explanation should be accepted. The presence of unripe reticulocytes with a structure possibly indicating a scar from the stalk after the budding is also interpreted in the same way. The paper should be read in the original by all those who are interested in the problem of erythrocyte formation.

J W

THE HISTOGENESIS AND DIAGNOSIS OF THE OSSEOUS TYPE OF GAUCHER'S DISEASE. M. Block and L. O. Jacobson. From the department of Medicine, University of Chicago, Chicago, Illinois. Acta haematologica 1: 165-177, 1948.

The diagnosis of Gaucher's disease depends upon the demonstration of the glucose containing cerebroside or of the characteristic Gaucher cell. The disease can be diagnosed before the appearance of hepatomegaly or splenomegaly.

The study of properly prepared sections of the bone marrow is a more certain method of diagnosis than the study of sternal puncture smears. Gaucher's disease is not a disease of the reticulo-endothelial system. The reticular cells, osteoblasts, osteoclasts and fibroblast like spindle cells of bone and marrow are the source of the Gaucher cell. The Gaucher cell is morphologically distinct from the cells found in the other fat storage diseases. Evidence is offered that red cells in the marrow in Gaucher's disease are present outside of what is ordinarily conceived to be blood vessels.

C M

RETICULO-ENDOTELIOSIS PALUDICA. (MALARIAL RETICULO-ENDOTHELIOSES) *C Martínez Mejía* Medicina (Revista Mexicana) 28 417, 454 459 481 1948

The paper begins with an anatomic description of the reticulo-endothelial system (issue 565), followed by a discussion of its physiology in relation with malarial fever (issue 566) and of its pathology in various infectious diseases (issue 567). Most of the photomicrographs have been taken from the collection of Dr. Soberon y Panra. The endo- and exoerythrocytic cycle of the plasmodium is stressed; the latter taking place in the reticuloendothelial cells where they remain in a latent state. This localization of the plasmodium, which appears to be most important, explains the therapeutic failures of most antimalarial drugs. She believes that the anemia of malarial fever is not only due to the destruction of the erythrocytes in the peripheral blood, but also to a block and degeneration of the hematopoietic organs. For the study of malarial reticuloendotheliosis she recommends the methods of Henry and Soberon.

R.M.S.

## HYPERSPLENISM

SPLENECTOMY IN THE RETICULOSSES. *L. J. Wills* From the Radcliffe Infirmary, Oxford, England. Acta med Scandinav Suppl 213, 352-364 1948. Studia in Honorem Einar Meulengracht.

The indications for splenectomy in 6 patients with reticuloses are discussed. In 2 patients the spleen was removed in order to alleviate pressure symptoms, in 2 for leukopenia, in 1 for thrombocytopenia. The effect was favorable in 5. In one patient a diagnostic splenectomy had no favorable effect.

J.W.

MYELOSCLEROSIS. A CASE WITH NON-MYELOID SPLENOMEAGLY AND AN ATTEMPT AT FINDING OUT THE PATHOGENESIS BY MEANS OF COMPARISON WITH RESULTS OF ANIMAL EXPERIMENTS. *H. C. Engell* From the Medical Department of the Frederiksberg County Hospital, Denmark. Acta med Scandinav 129 371 1947.

Splenomegaly and myelosclerosis were present in this patient and the author regards the changes in the spleen as nonleukemic. Splenectomy was performed at an early stage on the assumption that there was present a splenogenic inhibition of the marrow. The correctness of this explanation may be questioned but there was definitely an increase in leukocytes after operation. Later the patient died with anemia and thrombocytopenia but no leukopenia. The bone marrow showed increased fibrosis and the diagnosis was myelosclerosis. No extramedullary hemopoiesis was found.

J.W.

SPLENIC NEUTRO-THROMBOPENIA. *J. Bisbet* From the Roentgen Clinic of the Aarhus Municipal Hospital and the Radium Center for Jutland, Aarhus, Denmark. Acta med Scandinav Suppl 213 74-81 1948. Studia in Honorem Einar Meulengracht.

The history of a man 25 years old, who had suffered since childhood from recurrent stomatitis, angina and profuse nosebleeds is given. There was no anemia but the leukocytes decreased from 3,000 in January 1943 to 760 in February 1945. Platelets low. No signs of myeloblastosis in the bone marrow. Splenectomy gave prompt objective and subjective cure. Observation time two years. The patient's brother had typical symptoms of acute leukemia clinically. The possibility of a connection between the two diseases is pointed out.

J.W.

HYPERSPLENISM. SOME PRELIMINARY OBSERVATIONS. *W. Dameshek, and S. Estrin* From the Blood Laboratory of the J. H. Pratt Diagnostic Hospital, Boston, Massachusetts and the Department of Medicine, Tufts College Medical School, Boston, Massachusetts. Acta med Scandinav Suppl 213 106-119, 1948. Studia in Honorem Einar Meulengracht.

The paper gives an essence of Dameshek's ideas about hypersplenism with some illustrative case histories showing the importance of splenectomy.

J.W.

## BLOOD VOLUME

**DETERMINATION OF CIRCULATING RED BLOOD CELL VOLUME WITH RADIOACTIVE PHOSPHORUS** *R T Nisset, B Porter, W V Trautman, Jr, R M Bell, W Parson, C Lyons, and H S Mayerison* From the Laboratory of Biophysics Departments of Medicine, Surgery, and Physiology Tulane University School of Medicine and the Alton Ochsner Medical Foundation, New Orleans Louisiana *Am J Physiol* 155 226-231, 1948

The authors have presented a method for determining total circulating red blood cell volume by an isotope diluting technic using radioactive phosphorus. The authors point out that the subject to be studied is utilized for the labeling, that the counting is easy that determinations can be repeated, and that the rapid uptake and slow release of radioactive phosphorus by exposed red cells facilitates wide experimental application. Details of the method are too complex to put in abstract form.

R.C.C.

**COMPARISON OF RESULTS OF MEASUREMENTS OF RED BLOOD CELL VOLUME BY DIRECT AND INDIRECT TECHNIQUES** *H S Mayerison, C Lyons, W Parson, R T Nisset and W V Trautman, Jr* From the Departments of Physiology, Surgery, and Medicine and the Laboratory of Biophysics Tulane University School of Medicine and the Alton Ochsner Medical Foundation New Orleans, Louisiana *Am J Physiol* 155 232-238, 1948

The object of this experiment was to compare the results obtained with the dye method for determining red cell volume with the method utilizing radioactive phosphorus (*Am J Physiol* 155 226-231 1948). Concomitant measurements of red cell mass and plasma volume were made with the P 32 technic and the T 1824 method on 10 normal and 35 hospitalized patients. A standard correction factor of 0.915 was used to correct the hematocrit values for trapped plasma. Total blood volumes were calculated from the red cell volume and hematocrit and from the plasma volume and hematocrit. These values were compared with the total blood volume as calculated from the sum of the actually determined red cell and plasma volumes and showed satisfactory agreement. The data show that the plasma-dye hematocrit method is valid provided the corrected hematocrit value is used.

R.C.C.

**MEASUREMENT OF CIRCULATING RED-CELL VOLUME WITH METHEMOGLOBIN TAGGED CELLS** *J C Moore, O W Shadle and H C Lawson* From the Department of Physiology, University of Louisville School of Medicine, Louisville Kentucky *Am J Physiol* 155 322-329 1948

Circulating red cell volumes were determined on splenectomized barbiturized dogs by means of the conventional dye method and also by injecting a suspension of red cells containing large amounts of methemoglobin. The latter method always gave values that were lower than by the dye method even after corrections were made for the injected material.

R.C.C.

**DETERMINATION OF BLOOD VOLUME IN DOG BY MEANS OF VISUALLY LABELLED ERYTHROCYTES** *A Niget* From the Institut de Clinique et de Polyclinique Médicales Université de Liège *Quart J Exper Physiol* 34 123-128 1948

This study was undertaken to determine blood volume by means of labelling erythrocytes with Heinz granules by injections of phenylhydrazine. A known volume of labelled blood was injected intravenously into a dog and a blood sample removed five to forty minutes after the injection. By using a formula which is given the blood volume can be determined by counting the erythrocytes containing granules. The average circulating blood volume was found to be 66 cc per Kg of body weight.

R.C.C.

**EFFECT OF THE ADMINISTRATION OF ADRENALIN ON THE CIRCULATING RED CELL VOLUME** *W Parson, H S Mayerison, C Lyons, B Porter and W V Trautman, Jr* From the Departments of Physiology, Surgery and Medicine Tulane University School of Medicine and the Alton Ochsner Medical Foundation New Orleans Louisiana *Am J Physiol* 155 239-241 1948



This experiment was conducted to test the theory that there is a reserve of red blood cells in the spleen which can be utilized by the body in cases of emergency. Three normal adults, one patient with rheumatoid arthritis and one patient with hemolytic anemia were studied. After preliminary studies had determined the normal plasma and red cell volumes (by the dye and radioactive phosphorus methods) normal peripheral and body hematocrits and protein level each person was injected with 1 mg. of adrenalin. One man who expected adrenalin was given saline. This amount of adrenalin did not result in any significant changes in the plasma or red cell volumes. The authors conclude that if sympathetic stimulation or adrenaline influence these functions the effect must be very slight and of no real significance as an emergency response.

R.C.C.

## BLOOD COAGULATION AND HEMORRAGIC DISEASES

THE EFFECT OF NITROGEN MUSTARD AND X IRRADIATION ON BLOOD COAGULATION. L. O. Jacobson, E. K. Marks, E. Gasson, J. G. Allen and M. H. Block. From the Biology Division of the Argonne National Laboratory and the Department of Medicine, University of Chicago. Chicago, Illinois. J. Lab. & Clin. Med. 33: 1566-1578, 1948.

The administration of 3 or 4 mg. of nitrogen mustard per kilogram of body weight to rabbits produced a prolongation of the clotting time. The same syndrome was produced in human beings after therapeutic doses of this drug. The amount of protamine necessary to produce clotting in the heparin tolerance test was increased and the prolonged clotting time and decreased heparin tolerance were reversible with antihypertensive substances. The values for calcium, prothrombin and fibrinogen were normal. The platelets were reduced but prolongation of the clotting time took place prior to a significant reduction in platelets. The authors conclude that the anticoagulant present in the blood is probably heparin or a heparin-like substance.

G.E.C.

QUANTITATIVE STUDIES ON THE COMPARATIVE ACTIVITY OF CALCIUM AND CHEMICALLY RELATED IONS ON THE COAGULATION OF BLOOD. M. Stefanini and A. J. Quick. From the Department of Biochemistry, Marquette University School of Medicine, Milwaukee, Wisconsin. Am. J. Physiol. 152: 389-396, 1948.

Studies were made on the blood of man, dogs and rabbits. All calcium was removed from blood by treatment with Amberlite IR 100. The blood was then treated with varying concentrations of calcium, barium, strontium and magnesium to test the effects on coagulation. The optimal amount of calcium for coagulation was found to be the same as is found in blood normally. All the above mentioned elements have an inhibitory action on coagulation when increased above the optimal level.

R.C.C.

PLATELET EXTRACTS, FIBRIN FORMATION AND INTERACTION OF PURIFIED PROTHROMBIN AND THROMBOPLASTIN. A. G. Warr, J. H. Fabry and W. H. Seegers. From the Department of Physiology, Wayne University College of Medicine, Detroit, Michigan. Am. J. Physiol. 154: 140-147, 1948.

The object of this experiment was to determine the exact role of the platelets in blood coagulation. This study was aided by the availability of purified preparations of a number of the principal factors which participate in clotting reactions. Bovine platelet extracts contain an accelerator of prothrombin activation and only a small amount of thromboplastin. This accelerator is in an active form and acts in a similar manner to serum A<sub>2</sub> globulin. It is apparently a protein. Bovine extracts also contain a factor which hastens the second stage of clotting. The authors postulate that platelets aid in the initial formation of thrombin by catalyzing the interaction of prothrombin and thromboplastin. This thrombin then activates the inert plasma A<sub>2</sub> globulin to its active counterpart, serum A<sub>2</sub> globulin, which acts as the principal accelerator of the first stage of clotting.

R.C.C.

HEREDITARY HAEMORRAGIC TELEANGIECTASIA AND ITS RELATIONS TO OTHER INBORN VASCULAR MALFORMATIONS. H. M. Cohn, London and F. E. Rosenthal, Leicester. Acta haematologica 1: 81-91, 1945.

A report of two cases of multiple hereditary telangiectases with recurrent hemorrhages (Rendu Osler) is presented and compared with other cases of external and internal types of this condition with special regard to multiple telangiectases of the nervous system and other inborn vascular malformations.

In the first case the developmental error was not restricted to the structure of the telangiectatic malformations but extended besides to a degeneration of the collagenous and elastic tissue of the skin outside the telangiectases.

The second case offered the rare combination with a venous hemangioma of the spinal cord, causing the symptoms and signs of a cauda-conus tumor. This coincidence speaks in favor of some relationship between both inborn vascular lesions indicating a common congenital disorder of the vascular system.

The genetic connections between inborn vascular lesions of the skin and the nervous system are discussed.

C.M

**THROMBOCYTHEMIA HEMORRHAGICA** *Ole Mortensen* From the Department of Medicine Kolding Sygehus Denmark *Acta med Scandinav* 129 547 1948

The occurrence of chronic bleeding (nose, stomach and post traumatic) in spite of very high platelet counts (3-6 million) is illustrated by a case history of a man of 60 who had previously suffered from polycythemia. The erythrocyte values were exactly 5 million at the time of investigation but the leukocyte count was high (max 50 000). The syndrome is regarded as a malignant hyperfunction of the bone marrow of the same type as myeloid leukemia and polycythemia.

Obviously, the connection with polycythemia is quite intimate. A similar case is published by J. E. Holst, *Acta Medica Scandinavica* 130 507, 1948.

J.W

**THREE CASES OF POLYCYTHEMIA WITH FIBRINOPENIA** *S. E. Björkman* From the Medical Clinic of Akademiska Sjukhuset, Uppsala Sweden *Acta Med Scandinav* 129 471 1948

The author describes 3 cases of polycythemia with a bleeding tendency. Two of these cases had platelet-counts around 500,000 (normal value with the technic used 300 000). They also had low fibrinogen values but other polycythemics showed normal or increased fibrinogen values in spite of bleeding. A closer analysis of this symptom seems desirable.

J.W

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## NEWS AND VIEWS

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### JOSÉ ORIA

**I**N JULY 1948, Dr José Oriá, leading hematologist of Brazil and one of the Contributing Editors to this journal from Latin America, died in São Paulo, victim of a rare neoplasm. He was 43 years old and at the height of his professional career.

In Brazil, particularly in São Paulo, we owe a great debt of gratitude to those European physicians who, in response to the plea from the government of Brazil, came to the country and helped raise the standards of medical education. However, in the field of hematology there were no preceptors and José Oriá had to start completely on his own resources. He graduated from the Faculty of Medicine of

São Paulo in 1928, which was only fifteen years after the founding of the medical school. He was introduced to the study of morphology by the late Professor Bovero, an Italian disciple of Waldeyer, who organized the anatomy department of the medical school in São Paulo. Dr. Oria profited from the teachings of the Italian Hematologic School and in the twenty years that he worked and investigated, he contributed much to the advance of hematology in Brazil. In the last five years of his life he gave great impetus to cytochemistry and cellular enzymology. He founded the basis of modern cytology in the medical school of São Paulo. While organizing his department and arranging for original research he developed the fatal malignant disease which caused his death before the culmination of his carefully laid plans.

All scientific workers, especially physicians interested in morphology and hematology, deeply regret the loss of the man who was considered a corner stone of scientific thought in São Paulo. He had a brilliant, lucid and artistic mind and was endowed with keen powers of observation. He had the qualities of a patient and curious investigator, and was always bringing out cytologic details to the untrained eye of the student. He loved teaching in the objective way, side by side with his students. His comments often gave a rich view of the background and biologic significance of a simple slide.

Any one who ever knew José Oria has felt the loss of a friend, adviser and master.

MICHEL JAMIRA  
Hospital das Clinicas  
São Paulo, Brazil

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#### CONGRESS OF THE SOCIÉTÉ INTERNATIONALE EUROPÉENNE D'HEMATOLOGIE

This year's Congress of the Society (founded 1947) will take place at Montreux, Switzerland on September 15-17, 1949. The Chairman will be Prof. Chevallier (Paris), Prof. Lambin (Louvain) and Prof. Di Guglielmo (Naples).

The principal subjects to be discussed are (1) Hemolytic anemias (main report Prof. Heilmeyer) (2) Substances with inhibitory effects on mitosis and cytostatic action (main report Prof. Haddow, London, and Prof. Dustin, Brussels).

The time limit will be 10 minutes for short reports and 5 minutes for discussions. These are to be given either in French, German or English.

Members of the International Society of Hematology are cordially invited for participation at this Congress. Announcements for participation in the European Congress are to be sent as soon as convenient to the Secretary of the Society, Dr. S. Moeschlin, Medizinische Universitätsklinik, Zurich, Switzerland.

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# BOOK REVIEWS

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*Hemolysis and Related Phenomena* By ERIC PONDER New York, Grune & Stratton 1948 \$10 398  
Pages 69 Illustrations

This book will be welcomed by new readers as well as by the many who are familiar with the author's 1934 monograph *The Mammalian Red Cell and the Properties of Hemolytic Systems* of which the present volume is distinctly more than an expansion and modernization. Although as the author states, he has been taxed with talking of the red cell as a microcosm the range of his discussion from general physiology and biophysics to the problems of hemolytic anemias in man justifies his point of view particularly as he speaks from a direct and mature experience with many of the problems. The book is divided into seven chapters and contains four appendices and an up-to-date set of references as well as information derived from unpublished experimental work of colleagues. Those who, like the reviewer, are sufficiently illiterate in the language of quantity as not to read the calculus without a dictionary will have difficulty with some of the mathematical presentations, although this is mitigated considerably by the numerous graphic representations. The reason for the extensive use of footnotes is obscure, but these *obiter dicta* are richly rewarding.

The first chapter is valuable in orienting the reader to the basic questions to be discussed and their order of presentation. The next two chapters of the book are concerned with shape changes of the red cells occurring without and with volume changes, respectively. The first set of circumstances permits simplification of the consideration of methods of measurement of the dimensions of the red cell. In the next chapter the disclosure that shape changes may be accompanied by volume changes as well raises the often debated question of whether the red cell is a baglike structure containing hemoglobin and other substances in solution or whether it has an internal structure with orientation of the molecules, as the author's discussion makes clear, is manifestly the case for the envelope. The arguments for both types of structure are well set forth and the importance of recognizing that spheroidicity of the red cells may be the result of a variety of processes is made clear.

The fifth chapter discusses extensively the kinetics of hemolysis as observed in the test tube not only from a quantitative point of view but also qualitatively with respect to the prolytic changes in the red cell and their implications concerning structure. In the next chapter, inhibition and acceleration of hemolysis are discussed. Because of the evidence that immunologic mechanisms are concerned in some of the clinical forms of red cell destruction the reader may well wish that more space had been devoted to antibody-complement systems.

The final chapter should be of particular interest to readers of *Blood* because of the description of the nature of hemolytic processes involved in disease states. Interest by students of human disease in the precise mechanisms of red cell destruction is a relatively recent development; their preoccupation heretofore being with clinical classification and estimation of the magnitude of the hemolytic process through studies of pigment metabolism. If the discussion in this chapter is less definitive than that in others, it is mainly because the difficulties inherent in the conduct of observations upon human material have led observers to diverse conclusions. Moreover, hemolytic phenomena in experimental animals and in vitro have not yet been subjected to sufficiently critical analysis to establish their relationship to the processes of disease. The material presented in Dr. Ponder's book will serve to emphasize this fact to clinical investigators and will bring together in useful form information of value to those concerned with more fundamental problems relating to the red cell and to the structure of cell membranes in general.

W. B. CASTLE

*Blood Transfusions* By ELMER L. DEGOWIN, ROBERT C. HARDIN AND JOHN B. ALSEVER Philadelphia and London W. B. Saunders Company 1949 pp. 587

There can be nothing but praise for this book. It fulfills an immediate need; it is written simply; it is thorough, and the description and picturing of the methods used in blood grouping technique are unparalleled. The work is furthermore a triumph of artistic book making, not the least of which is due to a series

of 200 remarkable line drawings illustrative of the technic used. Everything connected with transfusions is presented through the combined efforts of three authors and an artist and everyone concerned must be congratulated on having put out this outstanding addition to the literature of modern therapeutic methods and blood grouping phenomena.

WILLIAM DAMESHEK

*Experimental Immunochemistry* By ELVIN A. KABAT AND MANFRED M. MAYER. Springfield Ill. C. C. Thomas, Pages 567.

This excellent book fills a real need by bringing together for the first time the many techniques of physics and chemistry which are used and useful in the field of immunology. The authors describe these procedures clearly. Those most easily available and commonly employed such as the complement fixation reactions, are covered in careful detail with wise enumeration of technical pitfalls to be avoided. The more difficult or specialized methods such as electrophoretic analysis, are dealt with less fully though no less lucidly, the intention in such cases being to familiarize the immunochemist with the principles involved and to enable him to evaluate critically the data which appear in the literature. In each instance, not only is a technic described but the theory underlying it is presented and limitations are pointed out as background for interpretation of results.

In their preface the authors describe the arrangement of the book. Parts I and II contain a detailed treatment of immunological and immunochemical methods and their application with emphasis on the evaluation of results by the quantitative methods. In Part III are described a variety of chemical and physical methods and special procedures frequently used by the immunochemist. Part IV includes details for preparing a variety of substances of importance in immunochemical work. The only regrettable omission is that dealing with the use of ion-exchange resins in the fractionation of plasma proteins so recently described as to preclude its presentation here.

If one is to carp at anything it must be the illustrations. The halftone reproductions are at times dull and somewhat smudged. Some of the line drawings have been reduced so much as to make their lettering illegible. But this slight flaw cannot detract from the value of the work as a whole.

The field of immunology has enjoyed many fruitful years in which great progress has been made. It should be realized however that agglutinin, hemolysin, complement, etc. are relatively vague and poorly defined entities which need the technic of immunochemistry for their more exact definition. It is certainly along chemical lines that future immunologic advance will be made. Thus this book must come as a most welcome tool to the immunologist and hematologic investigator.

WILLIAM CROSBY, Major, M.C., U.S.A.

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---

## *Contributors to this Issue*

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### BENJAMIN ALEXANDER, M D

Visiting Physician and Associate in Medical Research Beth Israel Hospital Associate in Medicine, Harvard Medical School Boston Mass

### LUCY MALZONE

Research Hematological Technician Holy Ghost Hospital Cambridge Mass

### JOSE O DE ALMEIDA, M D

Fellow of the Rockefeller Foundation on leave from Faculdade de Medicina São Paulo Brazil

### JACK MASON, M D

Formerly Fellow in the Department of Hematology, Cook County Hospital, Chicago Ill

### JØRGEN BICHEL, M D

Radium Centre for Jutland Aarhus University Aarhus Denmark

### STACY R. METTIER, M D

Professor of Medicine University of California Medical School San Francisco Calif

### FRANK H GARDNER, M D

Assistant Resident in Medicine Thorndike Memorial Laboratory Boston City Hospital Boston Mass

### WILLIAM C MOLONEY, M D

Professor of Clinical Medicine Tufts College Medical School Assistant Director 1st and 3rd Medical Services (Tufts) Boston City Hospital Boston Mass Chief of the Medical Service and Director of Clinical Research Laboratory Holy Ghost Hospital Cambridge Mass

### ROBERT GOLDSTEIN, M D

Associate in Medicine Beth Israel Hospital Assistant in Medicine Harvard Medical School Boston Mass

### J GRANT HARRISON, M D

Lieutenant (jg) (MC) USNR Resident in Medicine Meadowbrook Hospital Nassau County N Y Formerly of Department of Internal Medicine U S Naval Hospital St Albans N Y

### STEVEN O SCHWARTZ, M D

Attending Hematologist Cook County Hospital Hematologist Hektoen Institute for Medical Research of the Cook County Hospital Professor of Hematology Chicago Medical School Chicago Ill

### HAROLD A LYONS, M D

Commander (MC) USN Chief of Research U S N H St Albans N Y Instructor in Clinical Medicine Long Island College of Medicine Brooklyn N Y

### CARL H SMITH, M D

Associate Professor of Clinical Pediatrics Cornell University Medical College Associate Attending Pediatrician New York Hospital New York N Y

### FRANK MALTANER, PH D

Division of Laboratories and Research New York State Department of Health, Albany N Y

### ANDRE DE VRIES, M D

Formerly Research Fellow in Medicine Beth Israel Hospital and Harvard Medical School Boston Mass Present address Hadassah Hospital Jerusalem

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# BLOOD

## *The Journal of Hematology*

VOL IV, NO 6

JUNE, 1949

### CHRONIC CONGENITAL AREGENERATIVE ANEMIA (PURE RED-CELL ANEMIA) ASSOCIATED WITH ISO-IMMUNIZATION BY THE BLOOD GROUP FACTOR A

By CARL H. SMITH, M D

MANY VIEWS have been advanced to explain the pathogenesis of aplastic and hypoplastic anemia. The causative factors include chemical and physical agents, infection, exhaustion of the bone marrow, and specific blood dyscrasias and malignant tumors with bone marrow replacement. When these factors have been eliminated a relatively rare group of idiopathic aplastic anemia remains whose etiology is unknown and for which a congenitally inferior bone marrow has been postulated. In the classification of constitutionally defective bone marrow may be included examples of the Fanconi syndrome,<sup>1</sup> a type of anemia which is frequently familial and occurs in conjunction with a number of congenital abnormalities, chiefly pigmentation of the skin and testicular hypoplasia. Estren, Suess and Dameshek<sup>2</sup> recently described as a case of Fanconi's syndrome an 11 year old American-born child in whom hypoplastic anemia was associated with pigmentation of the skin, deafness, skeletal deformities and congenital heart disease.

It is the purpose of the present paper to describe a case reported in part of a previous communication<sup>3</sup> in which the failure of the bone marrow was confined to erythropoiesis without simultaneous depression of the granulocytes or platelets or their precursors. In considering the pathogenesis of this group of blood disorders the terms aplastic, hypoplastic and chronic congenital aregenerative anemia require definition. Aplastic anemia is a chronic progressive disease, characterized by a simultaneous depression of the three principal cellular elements in the bone marrow and resulting in a peripheral blood picture of profound anemia, leukopenia, neutropenia and thrombocytopenia. During the course of the disease the bone marrow shows a progressive decrease in the total count so that the megakaryocytes eventually disappear, the myeloid elements and nucleated red cells are greatly reduced and the lymphocytes predominate in the smears. Cases with normal or hyperplastic bone marrows with the peripheral blood picture of aplastic anemia have been interpreted as a maturation arrest or bone marrow block.

Hypoplastic anemia differs from aplastic anemia in that the formation of red blood cells is impaired with lesser involvement of the granulocytes and platelets. Earlier reports such as those of Josephs<sup>4</sup> and of Diamond and Blackfan<sup>5</sup> emphasized

From the New York Hospital and the Department of Pediatrics, Cornell University Medical College, New York, N. Y. (Presented before The Society for the Study of Blood at the New York Academy of Medicine, May 27, 1948).

the feature of chronic progressive anemia and correlated with it a failure of erythropoiesis without an equivalent depression of the white blood cells or platelets. Although hypoplastic anemia implies a less severe course and occasionally a more hopeful outcome than aplastic anemia, the term has, nevertheless, been applied in recent years to intermediate conditions in which the three blood elements of the bone marrow are involved in variable degree. Reports of cases of hypoplastic anemia now range from those limited to a failure of red cell production<sup>6</sup> to those in which leukocytes and platelets are simultaneously depressed but to a lesser degree than the red cells. In many of the earlier reports of congenital hypoplastic anemia, descriptions of the bone marrow<sup>7</sup> revealed a marked reduction in the number of nucleated red cells as well as an increase in the number of primitive cells or hematogones and of eosinophilic leukocytes. Estren and Dameshek<sup>8</sup> have recently described as hypoplastic anemia, familial cases with generalized quantitative hypoplasia of all the elements in the bone marrow with the nucleated red cells in normal or elevated percentages. In one of their cases of severe anemia with an increased number of reticulocytes and thrombocytopenia, splenectomy resulted in moderate clinical and hematologic improvement.

The elucidation of the factors responsible for the causation of the variety of blood disorders now included in the general category of the aplastic-hypoplastic type of anemia will be facilitated by segregating those cases which possess similar clinical and hematologic features. One group that lends itself for separate consideration concerns those instances in which the failure of hematopoiesis is restricted entirely to the erythrocytes without impairment of leukocytes or platelet production. This condition involving solely red cell production has been designated as chronic congenital aregenerative anemia by Vogel, Erf and Rosenthal,<sup>9</sup> but a more descriptive term is that of pure red-cell anemia, employed by Lescher and Hubble<sup>10</sup> who contributed 3 cases of their own. This unusual feature, in which a single cell type is depressed, constitutes the cardinal feature of this hematologic entity, and is illustrated by the following case history.

#### REPORT OF CASE

A. K. a white male infant, was born three weeks prematurely on December 28, 1946. The infant was firstborn. The delivery was normal and the infant was well for four days. At this time jaundice appeared which deepened and did not finally disappear until the third to the fourth week. At 8 days the blood count was 78 per cent hemoglobin with 3.7 M. red blood cells and the next day the values were slightly lower. No study of the blood factors was undertaken and no transfusion was given. On March 1, 1947 at approximately 2 months of age the baby developed an upper respiratory infection with a mucopurulent discharge. The child was admitted to a local hospital where a blood count revealed a hemoglobin of 29 per cent and a red count of 1.3 M. per cu. mm. One transfusion of 125 cc. of blood was given, and the child was discharged on March 4, 1947.

Both parents were healthy and there was no history of any hereditary blood disorder. There had been no preceding pregnancies.

The infant was admitted to the Children's Clinic of The New York Hospital on March 8, 1947 because of a progressive anemia.

**Physical Examination.** The infant was well developed and well nourished and in no distress. There was no jaundice; the heart and lungs were normal; the spleen and liver edge were palpable at the costal margin. There were no petechiae or other manifestations of bleeding into the skin.

**Laboratory Examinations.** The blood count on admission (table 1) revealed a hemoglobin of 9.5 grams per 100 cc. RBC 3.5 M. The white cells numbered 13,000 per cu. mm. with 25 per cent segmented

polymorphonuclear leukocytes 68 per cent lymphocytes 3 per cent monocytes and 4 per cent eosinophiles. On March 13 the hemoglobin was 8.9 grams per 100 cubic centimeters, the volume of packed red cells was

TABLE 1.—Representative Blood Counts in Case A. K.

Date	Hemo- globin content	Red cells	Packed red cells volume per cent	White cells per cu. mm.	Neu- tro- phils	Lym- pho- cytes	Mono- cytes	Eosino- phils	Platelets	Reticu- locytes
1947										
	Gm. per 100 cc.	millions per cu. mm.			per cent	per cent	per cent	per cent	cu. mm.	per cent
March 8	9.5	3,500		13,000	25	68	3	4		
March 13	8.9		24						290,000	0.2
April 4	8.5	2,910	26							
May 3	8.0	3,500		8,500	32	64	16	0		
June 2	8.0	2,600		16,800	26	68	6	0		
July 3	7.5	3,200		9,500	48	47	2	0		
October 8	10.0	3,040	28	15,400	37	57	4	2	296,000	0
November 17	8.5	2,300		15,800	47	46	7	0		
1948										
April 9	7.0	2,550	26	16,100	17	75	7	1	Numerous	0
May 13	8.0	2,750	28	9,200	31	66	2	1	Numerous	0

TABLE 2.—Hematologic Data in Case (A. K.) Showing Persistent Depression of Erythropoiesis in the Course of Chronic Congenital Aregenerative Anemia

Bone marrow aspiration	March 14 1947	May 3 1947	Nov. 18 1947
Total nucleated cell count per c.mm.	132,500	143,000	154,000
Megakaryocytes per c.mm.	66	77	77
Mycloblasts	0	0.5	3.0
Myclocytes	12.5	11.0	19.5
Metamyclocytes	7.5	5.0	7.0
Polymorphonuclears non segmented	30.5	27.0	31.0
segmented	9.0	12.0	6.5
Lymphocytes	39.5	44.0	30.0
Nucleated red cells	1.0	0.5	0
Hematogones	0	0	1.5
Monocytes	0	0	1.5

Blood groups: Father, A Rh positive; Mother, O Rh positive; Infant, A Rh positive

	March 3 1947	April 7 1947
Maternal anti A agglutinin titer	1:128,000	1:640-1:1280

24 per cent; the platelets numbered 290,000 per cubic millimeter; the reticulocytes were 0.2 per cent; the bleeding time was 3 minutes 35 seconds and the clotting time 3 minutes.

*Blood Group Factors.* The mother's group was O Rh positive and that of the infant and father A, Rh

TABLE 3—Representative Blood Counts in Case K. H

Date	Hemo- globin con tent	Red cells	Packed red cells volume per cent	White cells per cu mm	Neutrophils	Lymphocytes	Monocytes	Eosinophils	Basophils	Additional data
1947										
	Cm per 100 cc	Mil lions per Cu mm			per cent	per cent	per cent	per cent	per cent	
October 16	11 5	2,580		7,400	61	34	2	2		65 nucleated red cells per 100 white blood cells
October 17	10 4	2,700		12,300	72	21	2	2	3	17 nucleated red cells per 100 white blood cells
October 29	9 7	3,000		7,400	25	74	1			1 nucleated red cell per 100 white blood cells
November 4	11 0	3,400								0 2% reticulocytes
November 5				18,950	28	63	2	5	1	1 myelocyte
November 8	9 2	2,900								0 2% reticulocytes
November 14	11 5	3,600								
November 16				11,050	29	63	3	2		
December 1	8 9									
December 2	7 5									
December 4				10,800	25	68	5	2		
December 9	12 6	3,600								
December 23	15 9		39							
1948										
February 3	12 0		40							
May 4	13 5		44							

TABLE 4—Hematologic Data in Case (K. H) Showing Temporary Depression of Erythropoiesis in the Course of Erythroblastosis Fetalis

Bone marrow aspiration	Nov 5 1947	Nov 16 1947	Dec. 4 1947
Total nucleated cell count per c.mm.	145,000	25,450	146,000
Megakaryocytes per c.mm.	11	11	22
Myeloblasts	2 5	2 0	0
Myelocytes	16 0	12 5	18 5
Metamyelocytes	6 5	4 5	2 0
Polymorphonuclears non segmented	23 0	6 5	19 5
segmented	9 5	13 5	3 0
Lymphocytes	16 0	37 0	20 5
Nucleated red cells	7 0	0	30 0
Hematogones	19 5	24 0	6 5

Blood groups Father, O Rh positive, Mother O Rh negative Infant, O Rh positive

Anti Rh antibody titer (blocking)	Oct. 17 1947	Oct. 27 1947	Nov 4 1947	Dec. 3 1947
Mother	1 512			
Infant	1 64	1 128	1 64	1 16

Treatment—9 transfusions—total 555 cc. blood from Oct. 16 1947 to Dec. 9, 1947

positive. The clinical course and hematologic features in the infant appeared to be similar to those of mild erythroblastosis fetalis perhaps caused in this instance by isoimmunization of an Rh positive type O mother by an A offspring. Tests of the infant's saliva showed him to belong to the nonsecretor type.\* The mother's anti-A serum titer on March 3, 1947 was 1:128,000† and on April 7 it had dropped to a level of 1:640 to 1:1280.\* At no time was anti-A agglutinin detected in the infant's serum and his red cells gave a negative Coombs test. In cases of this sort it is necessary to exclude the possibility that other rare factors may be responsible for the isoimmunization. However, antibodies in the mother's serum for the five Rh Hr antigens in bloods of type Rh<sub>1</sub>Rh<sub>2</sub> could not be demonstrated.\* Consequently, the fact that the infant was of the nonsecretor type constitutes strong support for the role of isoimmunization by the factor A.

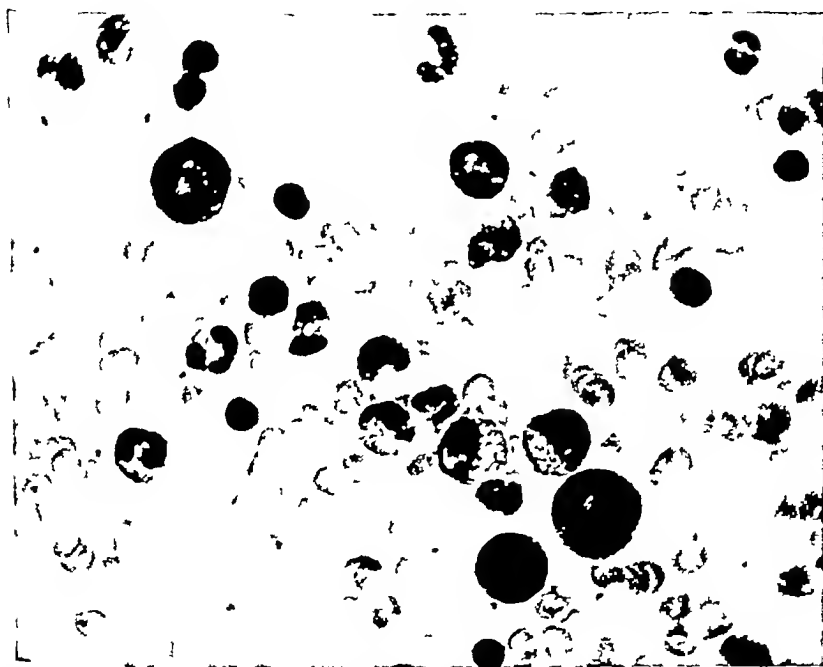


FIG. 1.—(Patient A. K.) Smear (X850) from sternal aspiration of November 18, 1947 (see table 1) showing polymorphonuclear leukocytes, myelocytes, metamyelocytes, lymphocytes and hematogones. Note absence of nucleated red blood cells.

*Course in Hospital.* Between March 8, 1947 and November 17, 1947, there were nine hospital admissions in each instance for treatment of progressive anemia. Eighteen transfusions were given at first employing O blood to which the Witebsky A and B substances were added and later with compatible Type A Rh positive whole blood. From December 16, 1947 until the present blood has been given at intervals of approximately 3 weeks in the Outpatient Transfusion Clinic at The New York Hospital. Growth and nutrition have been normal in every respect and susceptibility to infection has not been increased. There has been no enlargement of the liver, spleen or lymph nodes.

Tables 1 and 2 summarize the more significant blood serologic and bone marrow studies. Early in the

\* These tests were carried out by Dr. Philip Levine.

† Titration by Dr. Harry Wallerstein.



course the peripheral blood revealed evidences of macrocytosis and anisocytosis, later the red blood cells were normochromic and normocytic. The hematologic data confirmed the failure of erythropoiesis without involvement of the granulocytes and platelets or their bone marrow precursors.

### DISCUSSION

From the age of 2 months, when thorough hematologic studies were initiated, until the present age of 17 months, examination of the peripheral blood and bone marrow have consistently shown that the defect in hematopoiesis was confined to a failure of red cell formation. This anemia, in which granulocyte and platelet formation are unaltered, may be rightfully termed pure red cell anemia.

The only causative factor for the anemia that can be postulated in this case is the possibly injurious effect upon fetal erythropoiesis of the anti-A agglutinin elaborated by the mother in an incompatible pregnancy. The evidence for erythroblastosis fetalis in this infant is based on the history of jaundice and anemia noted in the first week of life. The relationship of erythroblastosis fetalis to sensitization by A and B agglutinogens in Rh positive mothers has been demonstrated by many observers<sup>11-18</sup> and its occurrence in this type of immunization in the firstborn has been pointed out by Wiener.<sup>17</sup>

While the blood disorder at the onset of this patient's illness can probably be safely classified as erythroblastosis fetalis, the relationship of the anti-A agglutinin to depression of the erythropoietic centers and continuance of the anemia require further elucidation. In 2 cases observed by Wiener,<sup>18</sup> sensitization by the A B agglutinogens was associated with an aregenerative anemia. Recent studies<sup>19</sup> which relate structural defects at birth to disturbances in the fetus may possibly be extended to include fetal anomalies of blood formation originating during critical periods of hematopoietic function. It is conceivable that erythropoiesis in the fetus may be impaired by prolonged reaction with an antibody in high titer against its own red cells in the course of an incompatible pregnancy. Levine<sup>21</sup> pointed out that the A and B blood agglutinable factors can be demonstrated in the fetus between the second and third month and suggested the possibility of early isoimmunization in the first months of fetal development. It is possible, therefore, that prolonged exposure of the red blood cells and their precursors during a vulnerable period of fetal life by the anti-A agglutinin may be responsible not only for the anemia at birth but for its persistence in the neonatal period.

In the group of hemolytic anemias which includes erythroblastosis fetalis, examination almost uniformly reveals hyperplasia of the bone marrow. Potter<sup>22</sup> has pointed out, however, that in some instances the bone marrow in erythroblastosis is either normal or hypoplastic, and that the latter state may account for the progression of the anemia. Diamond<sup>24</sup> states that bone marrow hypoplasia constitutes almost a uniform complication of any severe hemolytic anemia in the newborn. After the second or third week of life the infant, even after receiving multiple transfusions, often develops a relatively aplastic stage. It should be pointed out that Shapiro and Bassen<sup>25</sup> have shown that in full-term infants a marked drop occurs normally in the erythroid elements of the bone marrow at the end of the first week of life. It would be expected, however, that except for unusual circum-

stances, the increased hemolysis characteristic of erythroblastosis should result in a hyperplasia of nucleated red cells even at one week of age. In a series of fatal cases of erythroblastosis occurring at The New York Hospital, the bone marrow was recorded as showing hyperplasia in each instance.

To test further the hypothesis that erythropoiesis may become depressed in the course of erythroblastosis fetalis, bone marrow aspiration was carried out in several instances of this disease during the period of protracted anemia. Individual examinations have shown a decreased percentage of nucleated red cells in several cases, regardless of whether treatment consisted of subtotal blood replacement or multiple transfusion. In one case in which successive bone marrow aspirations were obtained (K. H., table 3), the mother was Rh negative and the infant Rh positive. This patient was also firstborn and this circumstance could be explained by a series of transfusions received by the mother before the birth of the child. Tables 3 and 4 demonstrate the temporary depression of erythropoiesis during the progress of the anemia in which nine transfusions of blood were required to maintain normal blood levels before recovery set in.

It appears, therefore, that the temporary failure of erythropoiesis occurring in erythroblastosis may conceivably be related to the exhausting effects of persistent hemolysis in this disease, or from the suppression of the erythropoietic centers in the bone marrow or in other fetal organs of blood formation by anti-A, anti-B, or anti-Rh agglutinins in susceptible individuals. It is possible that in the case of chronic congenital aregenerative anemia described in this paper the depression of erythropoiesis may have persisted from fetal life or from the period immediately following the newborn period as illustrated in the case of K. H. It should be pointed out that the high anti-A agglutinin titer detected in the maternal serum nine weeks after the birth of the infant may be an exaggeration of the actual agglutinin titer that was operative during fetal life. Boorman, Dodd, and Mollison<sup>26</sup> have shown the peak immune anti-A isoagglutinin titer produced in the maternal serum in response to stimulation by a group A or AB foetus, was not attained in the majority of cases until ten to twenty days after delivery.

The hypothesis that a prolonged depression in red blood cell production may result from an antibody specifically directed against the red cells in fetal life or during the early neonatal period and of a sufficient intensity to produce a chronic anemia extending into later infancy and childhood requires more extensive support. It should be emphasized that the concept offered to explain the mechanism of the anemia in this case is not expected to provide a uniform explanation for the pathogenesis of all cases of this entity. Although the circumstances noted in this patient may be unique, they afford a basis for further investigation of the causation of this unusual blood dyscrasia.

#### SUMMARY

Chronic congenital aregenerative anemia describes a pure red-cell anemia in which the failure of hematopoiesis is restricted entirely to the erythrocytes without simultaneous impairment of leukocyte or platelet production. The separation of this entity from the category of the increasing number of cases designated as

hypoplastic anemia will facilitate a more direct examination of the factors involved in its pathogenesis

In the case described in this paper illustrating this condition, the onset of the anemia dated to the newborn period with the clinical and hematologic features of a mild type of erythroblastosis fetalis. The mother's blood group was O, Rh positive and that of the infant and father A, Rh positive. The anti-A serum titer in the mother reached a maximum of 1:128,000. The infant was shown to be a non-secretor. The patient, now 17 months of age, requires repeated transfusions to maintain normal blood levels. The bone marrow reveals a persistent depression of erythropoiesis but the platelet and granulocyte levels are entirely unaffected.

It is postulated that prolonged depression in red blood cell production may result from an antibody directed solely against the red cells in fetal life or from the early neonatal period. This concept finds substantiation in other cases of erythroblastosis in which temporary failure of erythropoiesis as confirmed by bone marrow studies is reflected in a state of protracted anemia.

#### REFERENCES

- <sup>1</sup> FANCONI, G. Familiäre infantile perniziösartige Anämie (pernizioses Blutbild und Konstitution). *Jahrb f Kinderh* 117: 257, 1927.
- <sup>2</sup> ESTREN, S., SUSS, J. F. AND DAMESHEK, W. Coenatal hypoplastic anemia associated with multiple developmental defects (Fanconi syndrome). Report of case. *Blood* 2: 85-93, 1947.
- <sup>3</sup> SMITH, C. H. Treatment of the anemias of infancy and childhood. *J Pediat* 32: 721, 1948.
- <sup>4</sup> JOSEPHS, H. W. Anæmia of infancy and early childhood. *Medicine* 15: 307, 1936.
- <sup>5</sup> DIAMOND, L. K., AND BLACKFAN, K. D. Hypoplastic anemia. *Am J Dis Child* 56: 464, 1938.
- <sup>6</sup> KOHLERT, C. O. Coenatal hypoplastic anemia. *J Pediat* 19: 662, 1941.
- <sup>7</sup> SMITH, C. H. The anemias of early infancy. Pathogenesis and diagnosis. *J Pediat* 16: 375, 1940.
- <sup>8</sup> ESTREN, S., AND DAMESHEK, W. Familial hypoplastic anemia of childhood. Report of eight cases in two families with beneficial effect of splenectomy in one case. *Am J Dis Child* 73: 671, 1947.
- <sup>9</sup> VOGEL, P., ERF, L. A., AND ROSENTHAL, N. Hematological observations on bone marrow obtained by sternal puncture. *Am J Clin Path* 7: 436-498, 1937.
- <sup>10</sup> LESCHER, F. G. AND HUBBLE, D. A correlation of certain blood diseases on the hypothesis of bone-marrow deficiency or hypoplasia. *Quart J Med* 1: 425, 1932.
- <sup>11</sup> BOORMAN, K. E., DODD, B. E. AND MOLLISON, P. L. The clinical significance of the Rh factor. *Brit. M. J* 2: 569, 1942.
- <sup>12</sup> KEISALL, G. A. Erythroblastosis due to A-B-O incompatibility. *M. J. Australia* 2: 236, 1944.
- <sup>13</sup> POLAYES, S. H. Erythroblastosis fetalis in mothers with Rh-positive blood. Report of six cases with comment on iso-immunization with the A and B agglutinogens. *Am J Dis Child* 69: 99, 1945.
- <sup>14</sup> —, AND OHLEBAUM, C. Erythroblastosis fetalis unrelated to the Rh factor. Report of nine cases suggesting isoimmunization of group O mothers by A children. *Am J Clin. Path.* 15: 467, 1945.
- <sup>15</sup> —, AND McNALLY, J. Jr. Isoimmunization with the A and B factors and its relation to hemolytic disease of the newborn. *Am J Clin Path* 18: 375, 1948.
- <sup>16</sup> HALPERIN, J., JACOBI, M. AND DUBIN, A. The Rh factor in obstetrics. *Am J Obst & Gynec.* 56: 326, 1945.
- <sup>17</sup> WIENER, A. S., SONN, E. B. AND HURST, J. G. Pathogenesis of erythroblastosis fetalis. III. Illustrative case histories of A-B sensitization. Studies on individual differences in human blood and their practical applications. Paper No. 1, 22 pp. July 15, 1946. Brooklyn, N. Y.
- <sup>18</sup> OROZCO, A. C. V. The A and B Factors as a possible cause of erythroblastosis. *Blood Special Issue No. 2, The Rh Factor* page 164, January 1948.
- <sup>19</sup> LITCHFIELD, H. R. AND DEMBO, L. *Pediatric Progress*. Philadelphia: F. A. Davis Company, 1948.

- <sup>20</sup> WARKANY J. Etiology of congenital malformations. *Advances in Pediatrics* 2: 1. 1947. New York, Interscience Publishers, Inc.
- <sup>21</sup> LEVINE P. BURNHAM L. KATZIN E. M. AND VOGEL, P. The role of iso immunization in the pathogenesis of erythroblastosis fetalis. *Am J. Ost. & Gynec.* 42: 925. 1941.
- <sup>22</sup> ———. Serological factors as possible causes in spontaneous abortions. *J. Hered.* 34: 71. 1943.
- <sup>23</sup> POTTER, E. L. Rh. Its Relation to Congenital Hemolytic Disease and to Intragroup Transfusion Reactions. Chicago: The Year Book Publishers, 1947.
- <sup>24</sup> DIAMOND L. K. Discussion of current problems regarding the Rh factor. *Blood* Special Issue No. 2, The Rh Factor, page 181, January 1948.
- <sup>25</sup> SHAPIRO L. M., AND BASSEN F. A. Sternal marrow changes during the first week of life. Correlation with peripheral blood findings. *Am J. M. Sc.* 222: 341. 1941.
- <sup>26</sup> BOORMAN K. E. DODD B. E. AND MOLLISON P. L. Iso-immunization to the blood group factors A, B and Rh. *J. Path. & Bact.* 57: 157. 1945.

## MEDITERRANEAN ANEMIA IN THE NEGRO

### A REPORT OF FOUR CASES AND THEIR FAMILIES

By STEVEN O. SCHWARTZ, M D , AND JACK MASON, M D

FROM an ill-defined symptom complex known as von Jaksch's anemia there emerged in 1925 a disease entity to which the eponym Cooley's anemia has since been applied.<sup>1</sup> In its classic form it is characterized by occurrence in people of Mediterranean origin, a familial incidence, mongoloid facies, splenomegaly, characteristic bone changes and bone x-ray changes, severe anemia notable early in life, and the presence of erythroblasts in the peripheral blood. Patients having Cooley's anemia usually die before the age of 12.<sup>2</sup> The condition has also been called erythroblastic anemia, Mediterranean anemia and thalassemia,<sup>3</sup> the last name apparently being quite ill chosen.<sup>18</sup>

Dameshek,<sup>7</sup> Wintrobe, Matthews, Pollack and Dobyns,<sup>24</sup> and Strauss, Daland and Fox<sup>21</sup> independently, and almost simultaneously, described a less severe form of anemia characterized principally by the presence of target cells, ovalocytes, poikilocytes, hypochromic microcytes, and stippled red cells in the peripheral blood. One of the most constant features of this condition is the increased resistance of the cells to lysis in hypotonic saline solutions. Abnormalities of the red cells may be present even in the absence of anemia and not infrequently the red cell count is higher than normal. The bones may show evidences of osteoporosis with cortical thinning. Frequently some degree of splenomegaly and jaundice are present. The names thalassemia minor, Mediterranean anemia, target cell anemia, and familial microcytic anemia have been given to this less severe form.

Originally it was felt that an important facet in the diagnosis of Mediterranean anemia was the racial origin of the patient, since the disease was thought to be limited to people of the Mediterranean basin. Abandonment of this concept is compelled, however, by the increasing number of reports describing the condition in individuals of other racial groups.<sup>26</sup> One of Cooley's original seven<sup>2</sup> cases was a mulatto child, but this case was later withdrawn by Cooley since the child's blood had an increased, instead of a decreased, hypotonic saline fragility which began at 5 per cent and was complete at 35 per cent, the child lacked the mongoloid facies and improved on a good diet.<sup>4</sup> Dameshek,<sup>8</sup> in discussing the relationship of Mediterranean target-oval cell syndromes to sickle cell syndromes, mentions a case of Cooley's anemia observed in a Negro child at the Mt. Sinai Hospital of New York. Both hematologic and bone changes were present, and repeated examinations of the blood for sickling were negative.

Lubitz<sup>16</sup> in 1945 described 9 cases of Cooley's anemia in which target cells

From the Hematology Laboratory and the Hektoen Institute for Medical Research of the Cook County Hospital, Chicago, Ill.

Aided by a grant from the United States Public Health Service for the study of the Sickle Cell Phenomenon and by a grant from the Wilson Laboratories.

occurred in the absence of anemia. The first of these was a 20 year old Negro admitted for mumps. Red cell count was 5,080,000 and hemoglobin 14.0 Gm. Hemolysis began at 0.38 per cent and was complete at 0.28 per cent NaCl solution. When repeated, the range was 0.32 per cent to 0.14 per cent. Blood films showed 67 per cent target cells with many variants, and but few normally appearing red cells, there was some anisocytosis, moderate macrocytosis and ovalocytosis. The average red cell diameter was 7.4 micra, with the target cells distributing themselves over the entire range of the Price-Jones curve. There was no evidence of active or latent sickling. The skull and long bones were roentgenologically normal. Lubitz did not consider this a case of Mediterranean anemia because of the race, but felt that it was a related condition. Stiles, Manlove and Dangerfield<sup>20</sup> reported the case of a 23 year old Negro in whom an anemia was found during an admission for pneumonia to an Army hospital. Hemoglobin values varied between 8.75 and 9.2 Gm, and erythrocytes between 4.65 and 5.33 million. Occasional basophilic stippling, target and oval cells, anisocytosis, poikilocytosis, microcytosis, and hypochromia were present. Red cell fragility ranged from 44 per cent to 18 per cent saline solution, being incomplete at the latter figure, while the control ranged from 44 per cent to 32 per cent. Urobilinogenuria was present 1:20 on three occasions. The test for sickling was negative. X-rays of the skull showed increased porosity of the parietal bones. Blood films obtained from the sister showed anisocytosis, some poikilocytosis, hypochromia, and numerous target and oval cells. The patient is described as having a typical negroid appearance, but his sister and mother, who were also treated for anemia, were very light in color. In explaining the patient's anemia the authors imply that one of the maternal great-grand parents was Italian, but offer no proof of this. Faber and Roth<sup>10</sup> describe a 6 year old Negro girl with anemia, splenomegaly and peripheral blood changes compatible with Cooley's anemia. No sickling of the red cells was demonstrable. The bone changes, while not extreme, were of a character also compatible with the disease. The only possible discrepancy was the erythrocyte fragility which was stated as beginning at 56 per cent and complete at 35 per cent, duplicating a normal control. No other members of the family were found to have any blood dyscrasias. The father and brother were in the Oklahoma state hospital for the Negro Insane. Speculation by the authors as to the origin of the patient's disease has no factual support. The case cannot be accepted as one of Cooley's anemia on the basis of the evidence presented.

It is the purpose of this communication to report a series of cases of typical Mediterranean anemia in the Negro.

#### REPORT OF CASES

*Case 1.* Julius M. This 51 year old Negro railroad porter, a bachelor, was apparently in good health until November 1945 when he was admitted to a hospital in Memphis, Tennessee, where he was told that he had pneumonia, heart trouble, and a large spleen and liver. He continued to have a cough with occasional slight hemoptysis and chest pain after being discharged from the hospital. In September 1946 he noted increasing dyspnea and swelling of his abdomen and legs, this being worst in the evening and disappearing during the night. A feeling of pressure in the left upper quadrant was present since leaving the hospital in 1945. For ten or fifteen years he had consumed an average of two quarts of wine and one to

two pints of whiskey a week. There was no history of jaundice, gastro-intestinal hemorrhage, malaria, or exposure to lead. He had been stabbed in the abdomen in 1910 and was operated at the time.

Physical findings on admission: blood pressure 180/126, pulse 120, respiration 20, temperature 99.4 F rectally. Head: typical negroid appearance with very dark skin. Eyes: sclerae muddy, no definite jaundice, pupils round and equal, react to light and accommodation. Fundi: grade II retinopathy. Ears: nose, mouth, and throat essentially negative. Neck: some venous distention. Lungs: a few basal rales on both sides. Heart: enlarged to the left with the apex beat felt in the anterior axillary line in the sixth inter-space; no murmurs, a gallop rhythm noted when he was first seen. Abdomen: somewhat distended, a left rectus scar present. Spleen: firm, lower tip 15 cm below the costal margin. Liver: firm and smooth, edge three cm below the costal margin. No palpable peripheral lymph nodes. Multiple old healed ulcers on the legs. Normal reflexes. Rectal examination was negative.

TABLE 1—Summary of laboratory findings in Case 1 and relatives available for study. Since considerable difficulty was encountered in getting the relatives to submit to studies, obvious discrepancies are present which are designated by question marks.

	Patient Julius	J. M. (twin brother)	E. M. (younger brother)	A. M. (sister)
RBC	5.2 (average)	6.26	5.39	5.59
Hgb	71% (average)	113% (?)	87%	89%
Hematocrit	40%	43%	42%	40%
WBC	9,000	6,550	7,950	8,000
Nucl. RBC	1 to 2/100 WBC			
Target cells	90% or more	Very many	Approx. 50%	Occasional
Macrocytosis*	Present		Present	
Hypochromia	+++	++	+	
Poikilocytosis	Present		Present	
Anisocytosis	+++	++	++	
Reticulocytes	1 to 5%	1.1%	Less than 0.5%	0.4%
RBC fragility	0.36% to 0.01%	0.42% to 0.20%	0.55% to 0.24%	0.46% to 0.15%
Sed. rate	0 mm./hr.	0 mm./hr.	8 mm./hr.	12 mm./hr.
Sickling	Negative	Negative	Negative	Negative
Serum bilirubin	1.4 mg %	6 mg %	4 mg %	1.0 mg %
Serum iron	0.60 micrograms	0.55 micrograms	0.25 micrograms	0.30 micrograms
Urine				
Urobilinogen	++	++	+	Trace

\* Macrocytes noted were large thin cells.

Question marks refer to questionable findings which could not be rechecked.

Urine: albumin ++, sugar negative, specific gravity 1.014, urobilinogen ++ (repeatedly). Blood counts: see table 1. Total protein 7.3 Gm per 100 cc, with 4.3 Gm albumin and 3.0 globulin. Icterus index 9, NPN 29. Total cholesterol 210 mg per cent. EKG: Left axis shift. Abnormal graph compatible with coronary insufficiency. X-ray of the chest essentially negative. X-rays of femur, humerus, and hands—within normal limits. The skull had a few small discrete round, radio-luencies which are presumably due to Pachionian bodies. Multiple myeloma was considered a remote possibility. The marrow obtained by sternal puncture was found to be moderately cellular; megakaryocytes were present in adequate numbers and appeared to be normal; nucleated RBC:WBC ratio was about 3:2; erythropoiesis was normoblastic and showed a right shift; granulopoiesis was intact. The findings were compatible with regeneration secondary to active chronic hemolysis.

The diagnoses of (1) hypertensive heart disease with decompensation and (2) hemolytic anemia (Mediterranean type) were made.

The patient received ammonium chloride, thiamin, hykinone, glaucophyllin, and ferrous sulphate during his stay. He gradually became compensated and was discharged to the clinic with instructions to take a high protein diet, brewer's yeast, and digitalis.

*Patient History and Physical Findings of Parents and Siblings (fig 1)* As far as the patient knew his father was a full blooded Negro who died at the age of 89. The mother was brown skinned, and possibly had some Indian blood. There were 9 siblings. Five had died: one at the age of 2 and one at the age of 6 of causes unknown, the third died at the age of 26 of pneumonia and did not seem to be the right color when he died, the fourth died at the age of 32 of malarial fever following a few weeks illness, the fifth died at the age of 46 of pneumonia. Three of the living siblings were examined.

J. M. Jr. age 51, a nonidentical twin of the patient. He was of a much larger and heavier build and had been a prize fighter in his youth. Questioning revealed that his eyes had been yellow when he was younger, but he had attributed it to the trauma of prize fighting. He was married. His three children were dead. One died at the age of 3 of colic, one at 5 of pneumonia, and third at 6 of a cold. Physical examination was nonrevealing.

E. M. age 45, male, no pertinent history, had one son, age 25, in the Army. Physical examination was nonrevealing.

A. M. age 34, female, had no children. Her history and physical examination were without significance.

One sister lived out of town and was unavailable for study.

Blood findings are noted in Table 1.

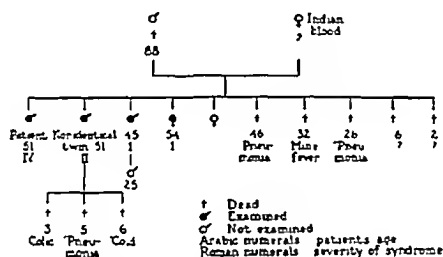


FIG. 1.—GENEALOGIC HISTORY OF CASE 1

The roman numerals refer to the type and severity of the individual's Mediterranean anemia. The classification is that of Dameshek and Limentani (table 5).

*Comment* The patient had no symptoms referable to his anemia. He presented himself because of cardiac decompensation, on the basis of a hypertensive cardiovascular disease. As in other types of hemolytic anemia numerous ulcers (healed) were present on the legs.<sup>9</sup>

No known Caucasian or Mediterranean admixture was present in the family. Of the four siblings examined, the patient had the most severe form of the disease, the nonidentical twin a less severe form, the younger and older siblings a still milder form. Whether the death of the siblings or the early death of the brother's three children had any relation to the disease under discussion is purely problematic.

*Case 2* Frank F. This Negro male was first seen in March 1941 (age 24) because of a cellulitis of the arm. A large hard spleen extending 4 cm. below the costal margin was found. The only other abnormal physical findings were small bilateral inguinal slightly enlarged axillary and palpable cervical lymph nodes. The liver was not enlarged.

The patient was born in Louisiana and lived there the first 20 years of his life. He had had fever at the age of 12 or 13 for which he had taken 666 and quinine. In 1940 he had a single chill but was able to return to work the following day. He used alcoholic beverages in moderation.

No malarial parasites were found after repeated adrenalin injections. The blood showed 63 per cent



(9.8 Gm) Hgb 3.95 RBC, 7,400 WBC, 74 polys (6 bands) 24 lymphocytes 1 monocyte 1 metamyelocyte. The red cells showed polychromatophilia ++ hypochromia + anisocytosis +, poikilocytosis +, with macrocytes present. The reticulocytes numbered 14 per cent and a rare nucleated red blood cell was seen. Wet preparations were negative for sickling. Hemolysis of red cells began at 0.38 per cent NaCl solution and was incomplete at 0.12 per cent. Urinalysis revealed no sugar or albumin. NPN 41 mg creatinine

TABLE 2.—Summary of hematologic findings in Case 2 and family

	Hgb	RBC	WBC	Retics	Target cells	Aniso	Hypo	Micro	Saline Fragility	Remarks
Frank F (patient)	83% average	5.10 average	7,500 average	5%	Almost 100%	+++	++		0.38% incomplete at 0.20%	Occ. nucleated red cells
Wife (second)	75%	4.55	5,900	2%	0		+	+	Normal	
M. L. age 10 female	85%	5.47	9,450	6%	About 10%	+	+		Normal	
F. Jr., age 7 male	87%	5.58	9,700	4%	About 10%	+	++		Normal	
Dorothy M. age 6 female	85%	5.69	10,900	4%	Almost 100%	++	+	+	42% to 15%	
Beulah M. age 4 female	80%	5.02	6,850	2%	10 to 20%	++	+	+	Not done	
Julia Ann age 5 mon, female	73%	4.84	18,750	4%	20 to 30%	++	++	+	Not done	

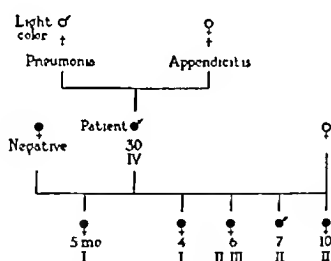


FIG. 2.—GENEALOGIC HISTORY OF CASE 2

The symbols are similar to those used on figure 1

1.3 mg. Sternal marrow was slightly hypercellular with a proliferation of the erythroid series. Discharge diagnosis was splenomegaly of unknown origin.

He was readmitted in September 1947. He had been working and well between admissions. In August 1947, however, he began to notice a dull ache in the left upper quadrant where he could feel a mass. The ache was brought on by a long walk or exercise and disappeared after a few minutes of rest. It was unrelated to meals and he felt as strong as ever before. Examination revealed a tall robust Negro with no significant physical findings other than the enlarged spleen which was felt nine cm. below the costal margin. The urine contained ++ to +++ urobilinogen. Kahn test was negative. Hematologic findings are shown in table 2. Serum bilirubin 1.35 mg. per cent. sedimentation rate 3 mm. per hour. hematocrit

43 per cent. The marrow was quite cellular, megakaryocytes were qualitatively normal but their number was relatively decreased, nucleated RBC/WBC ratio approximately 3:1, normoblastic erythropoiesis, large numbers of lymphocytes. The findings were again interpreted as compatible with chronic hemolytic anemia.

The patient's father died of pneumonia when the patient, an only child, was about ten years old and his mother died shortly afterward of appendicitis. He had been brought up by his paternal grandmother who was light brown and he remembered that his father was light too. He had 4 living children by his first wife, and one by the second (10, 7, 6, 4 years and 5 months). All the children were examined and none had abnormal physical findings. Sickling of the red cells could not be demonstrated in either the children or the second wife. The results of the hematologic studies are charted in table 2. One child (the third) had almost 100 per cent target cells and a slightly increased resistance to hypotonic saline solution (0.42 to 0.15). The second wife's blood was normal excepting for a slight iron deficiency. Both of the older children had a slight iron deficiency. Both of the older children had a slight erythrocytosis and the presence of target cells while the two youngest showed only target cells (fig. 2).

*Comment.* In spite of a fairly severe form of Mediterranean anemia this patient was never significantly incapacitated by his disease. Whether Mediterranean admixture was present is questionable, but that this is a possibility is suggested by the fact that the patient's father was light and the grandmother light brown. All the patient's children had mild forms of Mediterranean anemia, though the second wife was free of the trait as presumably was the first.

*Case 3.* Lillian N. This Negro female was born on April 18, 1935. In 1939 she was admitted to an other hospital because of an acute illness and upon recovery was discharged with the diagnoses of acute tonsillitis and pharyngitis, suppurative otitis media and sickle cell anemia.

In July 1942 she entered the Cook County Hospital with the admitting diagnosis of blood dyscrasia. Her presenting symptom was epistaxis of a week's duration. She had been having nosebleeds from time to time for two or three years but they had become more severe during the preceding year. There were no other complaints. She had had whooping cough at the age of 3, chicken pox at 4, and measles at 5. The only physical findings of significance were a systolic murmur at the apex of the heart and a freely movable enlarged spleen. Laboratory data: icterus index varied between 21-25, NPN 39, urines were negative for sugar, albumin and cells. X-rays of the skull showed considerable bony thickening with numerous sclerotic striations extending at right angles to the tangential line. These striations were particularly marked in the occipital area. There were also marked changes in the texture of the bones of the forearm and legs. The diagnostic possibility of Cooley's erythroblastic anemia was suggested. The blood counts are assembled in table 3. EKG was normal and showed a left axis deviation. Marrow at this time was reported as showing an erythroblastic proliferation compatible with a hemolytic process.

The patient's nosebleeds were few while she was in the hospital and she was discharged without specific therapy or a definite diagnosis.

She was readmitted in January 1946 with the diagnosis of splenomegaly. There was a history of chills, fever, jaundice, weight loss and headaches for one week. A slight jaundice, splenomegaly and a blowing systolic murmur over the base of the heart were found at this time. Laboratory data: Wassermann test negative, urine urobilinogen 0 to +++, total proteins 7.8, albumin 4.2, globulin 3.6, icterus index 13. Red cell fragility showed partial hemolysis at 30 per cent and was complete at 22 per cent. X-ray

The views of the tibiae, fibulae, radii, ulnae, carpals and proximal metacarpals show the following changes which are most marked in the metacarpals: thinning of the cortex, great prominence of the trabeculae, decalcification which is greatest in the spongiosa, some widening of the shafts of the bones especially in the metacarpals. The findings are probably all on the basis of hyperplasia of the bone marrow. Four views of the skull reveal marked atrophy of the outer table and generalized radial striations. The pictures are most like those described for erythroblastic (Cooley's) anemia although similar changes have been described in sickle cell anemia.<sup>11</sup> EKG: Rate 100, sinus rhythm, P-R interval 0.12 second, QRS 0.6 second, QRS upright in I and II, QRS<sub>3</sub> is notched and diphasic, S<sub>2</sub> present and T<sub>2</sub> inverted. Abnormal curve indicates myocardial damage. She was discharged as a case of sickle cell anemia.

In December 1947 she was seen in the Anemia Clinic. Physical examination revealed slightly icteric sclerae pallor of the mucous membranes and small shotty cervical, axillary and inguinal lymph nodes. The liver was felt 4 cm and the spleen 9 cm below their respective costal margins. A systolic murmur, most marked at the pulmonic area was heard over the precordium. Laboratory data +++ urobilinogenuria thymol turbidity 5.7 units stools negative for parasites blood negative for malaria. Sternal marrow aspiration revealed a very hypercellular marrow the megakaryocytes were relatively decreased nucleated RBC WBC ratio approximately 20:1, normoblastic erythropoiesis with a right shift intact granulopoiesis. X rays of the skull and long bones, as before, were typical of Cooley's anemia.

TABLE 3 — *Summary of representative blood counts obtained over a five year period in Case 3. It is to be noted that except following transfusions, she always appeared (pseudo) iron deficient and never showed sickling of the red cells*

Date	Hgb	RBC	WBC	Platelets	Parasites	Orthochrom. Normoblasts	Poly chrom. Normoblasts	Aniso	Poik	Poly	Hypo	Remarks
7/29/42	7.5 Gm. 45%	3.88	11,300			64/100 WBC	18/100 WBC	++	+++	+++	++++	No sickling
7/30/42		3.16		136,000								No sickling
8/5/42	7.5 Gm. 45%	3.98	6,850	291,000		39/100 WBC	16/100 WBC					Retic 4.7%
8/12/42	8 Gms., 48%	3.61	12,450		Negative for malaria							No sickling
1/15/46	9 Gms 54%	2.94	8,450			12/100 WBC	5/100 WBC	++++	++		+	
1/17/46	9 Gms 54%	3.08	9,550	323,000	Negative for malaria							No sickling
<i>Following Transfusion</i>												
1/22/46	13 Gm 78%	4.44	10,000									
12/24/47	49%	4.31	12,700			27/100 WBC		++++	+++	++	+++	Many targets. No sickling
12/24/47	6.0% Reticulocytes				Red cell fragility							
	Sed. Rate 4 mm corrected				trace 44%							
	Packed cell volume 30%				+ 42%							
	Serum Bilirubin 1.0 mgm				++ 36%							
	Serum Iron 0.85 mgm				+++ 28%							
					++++ 15%-10%							

The parents and siblings were examined as far as possible. The data are presented in Table 4.

The father and mother were both typically negroid in appearance. They were both about six feet tall and very well developed. Neither was jaundiced or had a splenomegaly. On the basis of the blood studies however they both had Mediterranean anemia.

The oldest child (D. N. male 16 years) had been diagnosed as having congenital heart disease at the age of 3. He had a to and fro murmur at the second left interspace with a rough systolic murmur at the apex. The blood picture was diagnostic of Mediterranean anemia.

The second child (G. N. male 14 years) had no abnormal physical findings. The blood findings however were those of Mediterranean anemia.

The patient (L. N. third child) had a severe anemia. blood and urine evidence of active hemolysis.

bone findings compatible with Cooley's anemia, hepatosplenomegaly, and stunting of growth. She is an example of Cooley's anemia.

The fourth child, (D, female, 10 years), had the same general appearance as her sister L. N. Prior to her birth, the mother had contracted syphilis and the child had been diagnosed as a congenital luetic. She was treated until the age of 9, when therapy was discontinued though her serologic tests for syphilis were still positive. She had a 'tower skull', hepatosplenomegaly and marked evidence of blood regeneration. She was also an example of Cooley's anemia.

The fifth child (W. N. male, 8 years), showed some target cells and an increase in urinary urobilinogen. No enlargement of the spleen or liver were demonstrable. Venipuncture was not allowed. This case represents the mildest form of red cell aberration seen in Mediterranean anemia.

The sixth child (R. N. female, 4 years) showed 30 per cent reticulocytes, some anisocytosis and hypochromia. The liver was felt 1.5 cm. below the costal margin but the spleen was not felt. Because no further studies could be performed it is not possible to ascribe definite significance to the high red count and low hemoglobin but in all probability this was a mild case of Mediterranean anemia. Data on the seventh and eighth children are insufficient for definite diagnoses. The blood film of the eighth child was however suggestive of Mediterranean anemia with great numbers of large thin cells, some target cells and anisocytosis being present. Both children had disproportionately high red cell levels for the hemoglobin values (fig. 3).

No history of exposure to lead or history of symptoms referable to lead poisoning, could be obtained from any of the family.

*Comment:* This patient had a typical Cooley's anemia as did a sister two years younger. As would be expected, on the basis of the assumption that this condition represents a homozygous trait, both parents showed evidences of a mild form of Mediterranean anemia. The other 6 siblings all had some stigmata of Mediterranean anemia. No evidence of Mediterranean admixture was found.

The patient (L. N.) and her sister (D. N.) were somewhat older and in much better clinical condition than the usual case of Cooley's anemia. However, Wolman and Dickstein<sup>26</sup> in their review of Mediterranean anemia list a number of reports of patients with moderately severe anemias and marked hematologic abnormalities, many of whom also had clinical and roentgenologic findings and yet survived beyond adolescence, and Daland and Strauss<sup>6</sup> report a case who survived to the age of 20 and gave birth to a viable child (who has Mediterranean anemia).

*Case 4 Frank D.* This 26 year old Negro male was first admitted to Cook County Hospital in July 1946 because of headaches, malaise, fever (six days), cough (two days), dyspnea (one day), and pain in the left chest especially when coughing (one day). Blood pressure 105/60, pulse 100, temperature 105.2 F, respirations 40. There was increased tactile fremitus, impaired resonance and bronchial breathing over the right lower lobe. The postauricular and inguinal lymph nodes were slightly enlarged. X-ray of the chest revealed only a prominent pulmonary conus. Kahn test was negative. The blood count, together with the count of the second admission is shown below. He was treated with penicillin and ferrous sulfate. His discharge diagnosis was broncho-pneumonia.

His second admission was in December 1947. This time he had a cold for two weeks with cough and fever and an urticarial rash for one day. There was no history of exposure to lead or of blood loss and the diet was adequate. His temperature was 102 F, respiration 24, pulse 120. He was well developed and acutely though not seriously, ill. The sclerae were slightly jaundiced. Small (0.5 cm.) bilateral discrete cervical nodes, a small (1 cm.) right epitrochlear, and discrete fairly firm inguinal nodes (3 cm.) were felt bilaterally. The liver was just palpable in the epigastrium. The spleen was firm and was palpable eight cm. below the costal margin.

Laboratory findings on this admission were as follows: ++++ urobilinogenuria, no glycosuria or

TABLE 4.—Summary of the laboratory findings in the family of Case 3. In this family both parents had mild Mediterranean anemia and therefore some evidence of the disease is expected to be present in 75 per cent of the offspring

	Hgb	RBC	Retic	WBC	Sickling	Serum Bilirubin mgm	Urine Urobilinogen	Cell Fragility to Hypotonic Saline	Blood Film	Remarks
Father age 41	94	7 00	1 9	12,100	Negative	2	++	trace 42% 36% 24% 20% 15-05%	Aniso + poik ++ poly ++ 62 suppld RBC, target cells 50%	Spleen not palpable Sed rate 2 mm (uncorrected) Packed cell volume 51%
Mother age 35	76	5 14	1 6	5,500	Negative	1	++	trace 44% 42% 36% 30% 24-20%	Hypo +, aniso +, about 50% target cells	Spleen not palpable
D N, male age 16	87	7 30	3 2	10,850	Negative	6	+	trace 42% 36% 28% 20% 15-10%	Poly ++, aniso with hypo +, poik +, 68 suppld cells 15% target cells	Sed rate 2 mm (uncorrected) 49% packed cell volume
G N male age 14	82	6 50	1 6	8,100	Negative		+++	trace 44% 42% 36% 28% 20-15%	Aniso ++ poik + miero + target cells about 50%	Spleen not palpable Sed rate 2 mm (corrected)
N L female age 12 (patient)	49	4 31	6 0	12,700	Negative		+++	trace 44% 42% 36% 28% 15-10%	27 NRBC/100 WBC aniso +++ poik +++ poly ++ hypo +++ many target cells	Sed rate 4 mm packed cell volume 30%

D N female age 10	55	5 16	9 6	12,900	Negative	1 0	+	+	+	+	+	trace	44% 40% 30% 20 10-05%	12. NRBC/100 WBC, aniso +++ poik +++ hypo +++ poly +, target +, stippling	Sclerae yellow bilateral small cervical nodes, spleen 11 cm down, liver 3½ cm down Sed rate 7 mm Hemat 33 vol %
W N, male age 8	89	5 31	1 2	4,400	Negative	+	+	+						Few target cells	
Ra N, female age 4	75	5 99	3 0	7,400	Negative	+	+							Aniso with hypo +	Liver 1½ cm down No jaundice Spleen not palpable
Rob N male age 2	78	6 54	1 0	15,350	Negative	+	+							Aniso with hypo ++	Spleen not palpable
J N, female age 1	67	5 58	4	9,770	Negative	?	+							Aniso ++, micro +, hypo +++ poik +, macro +, target ++	
Control fragil ity												trace + ++ +++ +++	48% 44% 42% 36% 28%		

albuminuria, NPN 38 Kahn test negative, agglutinations for typhoid paratyphoid and brucella negative, stool delayed + benzidine reaction for occult blood X-rays of the chest changes suggestive of an

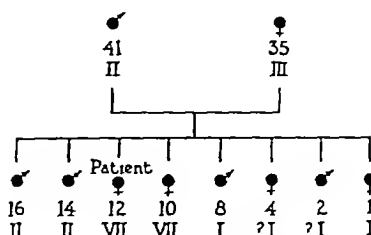


FIG 3—GENELOGIC HISTORY OF CASE 3

The symbols are similar to those used on figure 1

atypical pneumonia, of the skull granular appearance of both parietal bones probably the result of increase in the diploic markings but otherwise no bony changes characteristic of Cooley's anemia.

Blood counts				
Date	Hgb	RBC	WBC	
7/20/46	42%	3 25	13,900	Anisocytosis +++ polychromatophilia ++, hypochromia +, poikilocytosis +, target cells, 2 NRBC and 2 stippled cells/100 WBC
	(6.5 Gm.)			

12/20/47	45%	3 92	12,900	Anisocytosis +++ polychromatophilia + poikilocytosis +++ toxicity ++ 2 NRBC and 46 stippled RBC/100 WBC, Howell Jolly body
	(7.0 Gm.)			

Sickling negative in 24 and 48 hours  
Sedimentation rate 19 mm. (corrected) 12/24/47  
Hematocrit 31.5%  
Reticulocytes 4.8%  
Serum bilirubin 0.5 mg %

Red cell fragility	Trace	44%
	+	40%
	++	32%
	+++	20%
	++++	15-10%

Sternal marrow aspiration on December 12, 1947 revealed a very cellular marrow megakaryocytes were present in adequate numbers and appeared normal nucleated RBC WBC ratio was approximately 5:1 Erythropoiesis was markedly accelerated normoblastic in character and showed a left shift, mitotic figures in the erythroblast series were numerous granulopoiesis was not remarkable there was a moderate increase in plasma cells

**Family history** The patient was one of 11 children. He had an older brother in Chicago who was examined. Another brother had been killed in a train accident and one brother had died of liver disease. The mother and 7 sisters were living away from Chicago and were therefore unavailable for study. The father who was said to have been very light in color died (cause unknown) many years ago. Neither the patient nor his brother knew whether any of the sisters had been jaundiced or anemic. The brother's blood revealed RBC 5.69 Hgb 103 per cent WBC 14,150, reticulocytes 1.0 per cent hematocrit 43 per cent, urine urobilinogen + Serum bilirubin reported as 0. Fragility of the red cells began at 44 per cent NaCl solution and duplicated the normal control in the lower range. No enlargement of the liver or the spleen were demonstrable.

**Comment** The blood and marrow findings, the jaundice and the splenomegaly are diagnostic of a severe Mediterranean anemia. The brother (only sibling examined) does not have the disease (fig 4). Unfortunately, no one else in this large

family could be studied. What the father's light color signifies, as far as Mediterranean admixture in the patient's ancestry is concerned, is problematic.

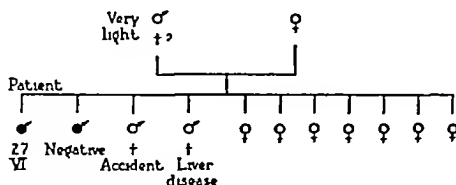


FIG. 4.—GENEOLOGIC HISTORY OF CASE 4  
The symbols are similar to those used on figure 1

### DISCUSSION

Cooley's anemia is the severest form of an aberration in red cell production and/or hemoglobin synthesis known as Mediterranean anemia. The mild form may be considered as being heterozygous for the trait, which is transmitted as an incomplete dominant factor<sup>6, 11, 17, 21, 22</sup> and whose presence can be demonstrated by hematologic studies. The severe form of the disease (Cooley's anemia) represents the homozygous form inherited from both parents. This is well exemplified in Case III. This relationship is not dissimilar to that occurring in sickle cell disease and sickle cell disease.

The shading from normal to Cooley's anemia is gradual and can be arbitrarily subdivided into mild, moderate, or severe forms or even into smaller groups, as suggested by Dameshek.<sup>6</sup> We have found his classification, which is reproduced here as table 5, quite useful, although intermediate forms and shadings from one group to the other occur no matter how small the subdivision. It soon must become obvious to those studying these cases that one must think of these conditions as more or less inconstant, rather than as arbitrarily fixed and readily pigeonholed.

The increasing number of reports dealing with the finding of sickle cell in Mediterranean peoples<sup>13</sup> and, on the other hand, the apparently not uncommon finding of Cooley's and Mediterranean anemias in Negroes, as indicated in this communication, leads to interesting speculation as to the consequences of the intermarriage of persons with sickle cell anemia and Mediterranean anemia. The two patients reported by Haden and Evans<sup>14</sup> may have been the offspring of such a union. They were of Sicilian descent, had anemia, splenomegaly, and sickling. Since they were 15 and 21 years of age, the presence of splenomegaly makes one especially suspicious of such possibility since marked splenic enlargement after the age of 7 in sickle cell disease is very uncommon.<sup>1</sup>

It is also worthy of speculation what the results of the crossing of other types of hemolytic anemias, with either Mediterranean or sickle cell anemias, would be. For example, we have recently seen a case of congenital hemolytic anemia in a Negro (11 other cases having recently been collected by Goodman and Cates<sup>15</sup>). What the result of the crossing of this disease with the above mentioned ones



would be is problematic and only the careful study of the families of all cases of atypical hemolytic disease will help clarify this problem

It is interesting, in retrospect, to note how often the diagnosis of sickle cell anemia had been made in our patients, notwithstanding the fact that sickling of the red cells was never demonstrated. However, it might even be conceivable that sickling of the red cells might be considered present in some cases, since in the moist chamber the naturally occurring poikilocytosis could, by the inexperienced, be mistaken for sickling. The number of these pseudo-sickle cells, however, does not increase even on prolonged standing.

The differential diagnosis between sickle cell anemia and Mediterranean anemia may be extremely difficult or even impossible on clinical grounds, and may depend entirely on the demonstration or lack of demonstration of sickling. The great

TABLE 5—Types of Mediterranean anemia (classification of Dameshek and Lawrence). It is to be noted that these divisions are arbitrary and overlapping between groups and findings is common.

	Hgb	Target oval or stippled cells	Spleno- megaly	Jaun- dice	Bone changes	Nucleated red cells
	%					
1 Congenital lepto and elliptocytosis	80+	+	o	o	—	—
2 Hypochromic erythrocytosis	80+	++	o	o	—	—
3 Hypochromic anemia	65-80	++	o	o	—	—
4 Hypochromic erythrocytosis with splenomegaly	65-80	++++	+	+	—	—
5 Congenital hemolytic target oval cell jaundice	50-65	++	++	+	+	—
6 Adult anerythroblastic type of Cooley's anemia	Less than 50	+	+++	++	++	—
7 Cooley's erythroblastic anemia	Less than 40	+	++++	++++	++++	++++

The common denominator of all the above types is the decreased hypotonic fragility, the presence of target and oval cells, and the lack of response to iron therapy.

similarity of the two conditions is emphasized in table 6. Whether these similarities imply an ethnologic or anthropologic relationship, we are unprepared to say, but the geographic proximity of the roots of these conditions certainly suggests such a possibility.

Malaria, especially in those patients who had spent some time in the South (as cases 1, 2, and 4) must be considered in differential diagnosis especially in view of the jaundice and splenomegaly. This condition was ruled out by careful examination of both blood films and marrows.

We were impressed by the excellent general health (with the exception of Case 3 and her sister with Cooley's anemia), the robust appearance, and the relative paucity of both ancient and recent history of illness which might have called attention to the underlying condition in this group of patients. This serves to underline the necessity for not only awareness but also of constant search for the

TABLE 6—Comparison between clinical and laboratory findings of sickle cell and Mediterranean anemias, showing for the most part, striking parallelism in the two conditions

	Sickle cell		Mediterranean anemia	
	Anemia	Trait	Cooley's anemia	Trait
Incidence	Familial		Familial	
Transmission	Hereditary		Hereditary	
Mendelian transmission	Dominant		Incomplete dominant	
Intermediate forms	Absent		Whole gradation	
Race	Primarily Negroes		Primarily Mediterraneans	
Pathogenesis	Inherited defect in red cell formation		Inherited defect in red cell formation	
Course and symptoms	Active disease Recurrent crises Death usually before age of 35	No symptoms	Gradation of severity from most severe (Cooley's) which is progressive and usually fatal before age of 12, to asymptomatic form	
<i>Physical findings</i>				
Pallor	Present	None	Marked	None
Jaundice	Present	None	Slight	None
Build	Underweight, long linear	Normal	Small squat	Normal
Head	Occasional tower skull	Normal	Large	Normal
Spleen	Large in childhood, atrophied in adulthood	Normal	Very large	Normal
Liver	Slight enlarged	Normal	Very large	Normal
Leg ulcers	Occasionally	Absent	Occasionally	Absent
Bone X rays	Ground glass and hair on-end appearance of skull Medullary widening and cortical thinning of long bones (variable)	Negative	Hair on-end appearance of skull Medullary widening and cortical thinning of long bones	Negative
<i>Blood findings</i>				
Anemia	Moderate to severe Usually normocytic normochromic	None	Severe Microcytic hypochromic	None
Oval cells	Present	Occasionally present	Present	Present
Target cells	Present	Present	Present	Present
Sickling of cells	Marked	Present	Absent	Absent
Hypotonic saline resistance	Increased	Normal	Increased	Increased
Nucleated red cells	Especially during crises	Absent	Numerous	Absent
Basophilic stippling	Present	Absent	Present	May be present
Reticulocytosis	Marked during crisis	Normal	Marked	May be slightly elevated
Leukocytosis	Marked during crisis	Absent	High	Absent
Bilirubin	Elevated	Normal	Elevated	Normal
Bone marrow	Hyperplastic with normoblastic proliferation	Normal	Hyperplastic with normoblastic proliferation	May be slightly hyperplastic

disease. By virtue of the partial dominance of the gene it is safe to assume that we are dealing with a condition whose recognition will henceforth occur more and more frequently.

We wish to re-emphasize the dictum previously advanced<sup>19</sup> that even though our patients had the typical negroid appearance of skin, hair, facies, etc., Caucasian admixture cannot be ruled out. This aspect of the problem is entirely academic, since the North American Negro is admittedly a hybrid group, but none-the less represents the type of patient in whom we are interested. Whether the condition exists among unmixed Negro races remains to be answered by those who have access to this type of material.

### SUMMARY

Four cases of Mediterranean anemia are reported in Negroes.

The hematologic and clinical findings of available relatives are presented.

The disease in the Negro resembles the condition as found in people of Mediterranean ancestry in every particular.

### REFERENCES

- <sup>1</sup> ANDERSON, W. W., AND WARE, R. L. Sickle cell anemia. *Am J Dis Child* 44: 1055, 1932.
- <sup>2</sup> COOLEY, T. B., AND LEE, P. A series of cases of splenomegaly in children with anemia and peculiar bone changes. *Tr Am Pediat Soc* 57: 29, 1925.
- <sup>3</sup> ———, WITWER, E. R., AND LEE, P. Anemia in children with splenomegaly and peculiar changes in the bones. Report of a case. *Am J Dis Child* 34: 347, 1927.
- <sup>4</sup> ———, AND LEE, P. Erythroblastic anemia. Additional comments. *Am J Dis Child* 43: 705, 1932.
- <sup>5</sup> DALAND, G. A., AND STRAUSS, M. B. The genetic relation and clinical differentiation of Cooley's anemia and Cooley's trait. *Blood* 3: 438, 1948.
- <sup>6</sup> DAMESHEK, W. Personal communication.
- <sup>7</sup> ———. Target cell anemia. Anerythroblastic type of Cooley's erythroblastic anemia. *Am J M. Sc.* 200: 445, 1940.
- <sup>8</sup> ———. Familial Mediterranean target-oval cell syndrome. *Am. J. M. Sc.* 205: 643, 1943.
- <sup>9</sup> ESTES, J. E., FABER, E. M., AND STICKNEY, J. M. Ulcers of the leg in Mediterranean disease. *Blood* 3: 302, 1948.
- <sup>10</sup> FABER, H. K., AND ROTH, B. M. Mediterranean anemia (Cooley) in a Negro girl. *J. Pediat* 28: 610, 1946.
- <sup>11</sup> GATES, R. R. *Human Genetics*. New York, The MacMillan Co. Vol. I 638, 1946.
- <sup>12</sup> GOODMAN, E. G., AND CATES, B. R. Case report. Congenital hemolytic icterus in the Negro. *Blood* 2: 480, 1947.
- <sup>13</sup> GREENWALD, L., SPIELHOLZ, J. B., AND LITWINS, J. Sickling trait in a white adult associated with hemolytic anemia, endocarditis and malignancy. *Am J M. Sc.* 206: 158, 1943.
- <sup>14</sup> HADEN, R. L., AND EVANS, FERRIS, D. Sickle cell anemia in the white race. *Arch. Int. Med.* 66: 133, 1937.
- <sup>15</sup> KRAFT, E., AND BERTEL, G. Sickle cell anemia. *Am J Roent & Radium Therapy* 57: 224, 1947.
- <sup>16</sup> LUBITZ, J. M. Target cells and other abnormalities of red cells in the presence of a normal erythrocyte count and hemoglobin content. *Am. J. Clin. Path.* 15: 305, 1945.
- <sup>17</sup> NEEL, J. V., AND VALENTINE, W. N. Further studies on the genetics of thalassemia. *Genetics* 32: 38, 1947.
- <sup>18</sup> NITTS, SAVAS. Letter to the Editor. *Blood* 3: 326, 1948.
- <sup>19</sup> SCHWARTZ, S. O., AND GORE, M. Pernicious anemia in Negroes. *Arch. Int. Med.* 72: 782, 1943.
- <sup>20</sup> STILES, M. H., MANLOVES, C. H., AND DANGERFIELD, C. D. Mediterranean anemia. Report of a case in a Negro. *Northwest Med.* 45: 23, 1946.

- <sup>21</sup> STRAUSS, M. B., DALAND G. A., AND FOX, H. J. Familial microcytic anemia. *Am J M Sc* 201: 30, 1941.
- <sup>22</sup> VALENTINE, W. N., AND NEEL, J. V. Hematologic and genetic study of the transmission of thalassemia. *Arch Int Med* 74: 185, 1944.
- <sup>23</sup> WHIPPLE, G. H. AND BRADFORD, W. L. Racial or familial anemia of children associated with fundamental disturbances of bone and pigment metabolism. *Am J Dis Child* 44: 336, 1932.
- <sup>24</sup> WINTROBE M. M. MATTHEWS E. POLLACK, R. AND DOBYNS, B. M. A familial hemopoietic disorder in Italian adolescents and adults. *J A M. A* 114: 1530, 1940.
- <sup>25</sup> ———. *Clinical Hematology*. Philadelphia, Lea & Febiger 2: 565, 1946.
- <sup>26</sup> WOLMAN I. J. AND DICKSTEIN B. Changing concepts in Mediterranean (Cooley's) anemia. *Am J M. Sc.* 112: 723, 1946.

# A RAPID SLIDE TEST FOR HETEROPHILE ANTIBODY IN INFECTIOUS MONONUCLEOSIS

By WILLIAM C. MOLONEY, M D, AND LUCY MALZONE

SINCE the discovery by Paul and Bunnell<sup>1</sup> in 1932 of the presence of heterophile antibodies in the sera of patients with infectious mononucleosis, there has been a great deal of investigation into the nature and production of anti-sheep red cell agglutinins.<sup>2</sup> With the advent of the Rh factor and the subsequent discovery of blocking<sup>3</sup> (incomplete,<sup>4</sup> or hyperimmune<sup>5</sup>) antibody new avenues of approach were opened to many perplexing problems concerning red cell antigenicity.

In 1945, Levine and Gilmore<sup>6</sup> reported the discovery of a blocking antibody in the serum of a patient with infectious mononucleosis. At this time attempts by one of us (W. C. M.) to disclose heterophile blocking antibody, using sheep and goat cells, were unsuccessful.<sup>7</sup> However, this work was carried out in England while in the Army Medical service and comparatively few cases were studied, moreover, the proper breed of goat was not obtainable. When knowledge of Diamond's slide method for Rh testing became available in June 1945, a modification of this test was employed to further search for the presence of blocking antibody in the sera of cases of infectious mononucleosis and other diseases. For the past three years this work has been carried out more extensively in civilian practice and since the slide method may have practical applications, its use is reported in this paper.

## METHODS AND MATERIALS

The test was carried out by mixing 0.1 cc. of defibrinated sheep blood on a glass slide with 0.2 cc. of serum to be tested. Tests were considered positive only if 3 plus to 4 plus macroscopic clumping occurred within 30 to 60 seconds. The heterophile antibody test was carried out on the same sera using the Paul Bunnell method.<sup>1</sup> A serum dilution of 1:128 was considered the lowest positive level. The sheep cells were preferably used fresh but defibrinated sheep blood kept at 5°C for two weeks gave reliable tests. Citrated, phenolized and 50 per cent saline sheep cell suspensions also gave good results with strongly positive sera. However, to avoid factors which might interfere with blocking antibody only defibrinated sheep blood was used in the slide tests reported in this paper. Serum was obtained in the usual fashion. Inactivation was carried out in a number of cases but for practical purposes this was found to be unnecessary. Sera kept in the icebox lost potency slowly while if stored in the deep freeze the heterophile antibody content was well preserved for long periods. The amounts of serum and cells used in the test made a definite difference in the agglutination reaction. A 2 to 1 proportion of serum to cells was found to give the most clear-cut test. All slide tests were carried out at room temperature. As described below the heterophile antibody in infectious mononucleosis is active at 37°C as well as at lower temperatures. In certain cases of cirrhosis and patients with hemolytic syndromes the antibody which gave a positive slide test at room temperature was inactive when the test was carried out at 37°C.

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From the Clinical Research Laboratory, Holy Ghost Hospital, Cambridge, Massachusetts and the Departments of Medicine and Pathology, Tufts College Medical School, Boston, Massachusetts.

Paper read at the annual meeting of the American Federation for Clinical Research, Atlantic City, N. J., May 4, 1948.

In part this study was supported by a grant from the American Cancer Society (Massachusetts Division).

## RESULTS

Tests were carried out on the sera of 473 individuals with various diseases and in this group were included a number of normal controls

*Infectious Mononucleosis* In this group there were 41 patients with definite hematologic and clinical evidence of the disease (see table 1)

Of these 41 cases, 34 were serologically positive by the Paul-Bunnell test at some time during the course of the illness. There were 6 cases in which repeated heterophile antibody titers were 1:8 or below and the slide tests were also negative in these individuals. In one case the heterophile antibody titer was 1:64 and at the same time the slide test was positive, otherwise the remaining 34 cases had titers of 1:128 or above with strongly positive slide tests. It was observed that in

TABLE 1.—*Infectious Mononucleosis*

Diagnosis	Total cases	Positive slide test	Positive Paul Bunnell test
Infectious Mononucleosis	41	35	34

TABLE 2.—*Diseases of the Liver*

Diagnosis	No. of cases	Positive slide test	Positive Paul Bunnell test
Cirrhosis (Laennec)	43	6*	0
Cirrhosis (biliary)	5	0	0
Cirrhosis (post infectious hepatitis)	5	1*	0
Acute infectious hepatitis	21	0	0
Total	74	7	0

\* Positive at room temperature, negative at 37 C.

following the disease along, as the heterophile titer in saline fell below 1:128 the slide tests became negative.

*Diseases of the Liver* The sera of 53 patients with cirrhosis of the liver and 21 patients with acute infectious hepatitis were tested (see table 2.)

In 6 cases of cirrhosis of the Laennec type and one of cirrhosis following infectious hepatitis, positive slide agglutinations of sheep cells occurred. However, in none of these cases were sheep cell agglutinins by the Paul-Bunnell method present in a dilution of 1:8 or above. On carrying out the slide test at 37 C, the agglutination disappeared. This is in contrast to the sheep cell agglutinins found in infectious mononucleosis which are active at 37 C as well as lower temperatures. It was concluded that the sheep cell clumping observed on the slide at room temperature in these cases was due to nonspecific cold agglutination.

Although most of the cases of infectious mononucleosis in this series gave positive cephalin-cholesterol flocculation, thymol turbidity and ZnSO<sub>4</sub> turbidity tests, there was no apparent correlation between the presence of heterophile anti-

body (either by slide test or by the Paul-Bunnell method) and the occurrence of these tests which indicate an alteration of the serum proteins. In keeping with the observation of others, positive heterophile antibody tests were not found in the cases of infectious hepatitis.

*Malignant Diseases* The sera of 58 patients with a variety of neoplastic disorders were examined for heterophile antibodies (see table 3).

In only one case was a positive slide test observed. This occurred in a patient with multiple myeloma but subsequently repeated tests on the same patient were negative. Sera from other patients with multiple myeloma have shown no increase in heterophile antibodies nor have slide tests been positive.

TABLE 3.—*Malignant Diseases*

Diagnosis	No. of cases	Positive slide test	Positive Paul Bunnell test
Cancer	40	0	0
Malignant lymphoma	5	0	0
Leukemia	7	0	0
Multiple myeloma	6	1*	0
Total	58	1	0

\* Nature of antibody not known.

TABLE 4.—*Normal Pregnancy and Cord Blood*

Diagnosis	No. of cases	Positive slide test	Positive Paul Bunnell test
Pregnancy	95	0	0
Cord blood	23	0	0
Total	118	0	0

*Normal Pregnancy and Cord Blood* The sera of pregnant women may show various positive flocculation tests. However, in 95 patients during pregnancy and in 23 cord blood specimens there were no positive heterophile antibody tests (see table 4).

*Hemolytic Disorders and Iso-immunized Women* This group of patients deserves special consideration and further studies are being carried out (see table 5).

In 7 patients with acquired hemolytic anemia there were 2 cases which gave positive slide tests. These 2 individuals had no increase in heterophile antibody by the Paul-Bunnell test. When the slide test was carried out at 37°C, no agglutination occurred. These two patients had very strong cold autohemagglutinins. Both had undergone splenectomy without improvement and subsequently one patient died and was found to have a bizarre myeloblastic leukemia. The other patient survived but has continued to show hemolytic anemia and no further underlying disease has been disclosed to date.

In the sera of 10 women heavily immunized in pregnancy by the Rh factor there were no heterophile antibodies found. There were 3 women strongly immunized

by fetal A<sub>1</sub> cells and one woman with anti-B agglutinins giving a positive serum dilution of 1:60,000. In none of these women were there positive heterophile antibody tests. However, a patient who is still under investigation has been of considerable interest. After this woman gave birth to her second baby the infant developed moderately severe hemolytic disease. The mother was O, Rh positive, the father was also Rh positive, A<sub>1</sub> A<sub>2</sub>—and the infant was A<sub>2</sub> O, Rh positive.\* The mother developed an Anti A<sub>2</sub> agglutinin which reached a positive dilution of 1:50,000. This serum also gave a positive slide test for heterophile antibody which did not disappear at 37°C and the Paul-Bunnell test showed a borderline

TABLE 5—*Hemolytic Syndromes and Iso-immunized Women*

Diagnosis	No. of cases	Positive slide test	Positive Paul Bunnell test
Acquired hemolytic anemia	7	2*	0
Anti Rh agglutinins	10	0	0
Anti A <sub>1</sub> agglutinins	3	0	0
Anti B agglutinins	1	0	0
Anti A <sub>2</sub> agglutinins	1	1	1†
Total	22	3	1

\* Became negative at 37°C.

† Border line positive dilution.

TABLE 6—*Miscellaneous Diseases and Controls*

Diagnosis	No. of cases	Positive slide test	Positive Paul Bunnell test
Miscellaneous	50	0	0
Controls	110	0	0
Total	160	0	0

positive dilution of 1:64. On absorption tests the antibody was absorbed by guinea pig kidney but not by boiled beef cells. This antibody was apparently related to the Forssman type rather than the variety of sheep cell agglutinins found in infectious mononucleosis.

*Miscellaneous Diseases and Controls.* Tests were carried out on the sera of patients with a variety of diseases. In this group were included 3 cases of serum sickness. None of these had positive heterophile tests although it should be expected that if strong enough, the heterophile antibodies of the Forssman type would give positive slide tests. Unfortunately, the only tests on these three cases were carried out on the 1st or 2nd day of the illness and later specimens of serum were not obtained for testing (see table 6).

In the sera of 110 normal individuals, there were no false positive tests.

\* The genotypes and specificity of the anti A sera were kindly determined by Dr. William Boyd of Boston University Medical School.



## SUMMARY AND CONCLUSIONS

The sera of 473 individuals were examined for sheep cell agglutinins both by the slide test and the Paul-Bunnell method. In this group there were 46 patients with positive slide tests and 35 of these individuals also had a diagnostic serum dilution test for heterophile antibody. In 11 cases the slide test was positive but the Paul Bunnell test gave very low serum dilution values. However, when the slide test was carried out at 37 C, it was negative in 9 of the 11 cases. In the remaining 2 instances, one patient had a Forssman type of antibody which gave a 1:64 titer in saline and the slide test was positive at 37 C. In the other case no studies were made on the effect of temperature and the nature of the agglutination reaction was unfortunately not determined.

Using human and bovine albumen, sheep serum and human AB serum absorbed with sheep cells as a diluent no evidence for blocking or hyperimmune antibody was discovered in the cases of infectious mononucleosis studied in this series. Moreover, of the 6 patients with negative serology but with strong clinical and hematological evidence for the disease, no blocking or hyperimmune antibody was disclosed by the slide test or by the use of absorbed human AB serum. The conclusion seems justified that blocking, incomplete or hyperimmune heterophile antibody must be rather uncommon in infectious mononucleosis.

In the use of the rapid slide test it has been pointed out that cold agglutinins, (which may be abolished by warming to 37 C) and Forssman antibodies (which may be absorbed by guinea pig kidney) can give positive results. However, diseases in which cold agglutinins are strong enough to give a positive slide test are relatively rare and the occurrence of Forssman antibodies of a strength likely to give a positive slide test would be decidedly uncommon. In any event unless further experience reveals more serious discrepancies, the rapid slide test as described in this paper seems to offer a practical screening test to detect clinically significant amounts of heterophile antibody in cases of infectious mononucleosis.

## REFERENCES

- <sup>1</sup> PAUL, J. R., AND BUNNELL, W. W. Presence of heterophile antibodies in infectious mononucleosis. *Am J M. Sci.* 183: 90, 1932.
- <sup>2a</sup> DAVIDSOHN, I. Serologic diagnosis of infectious mononucleosis. *J. A. M. A.* 118: 289, 1937.
- <sup>b</sup> STRAUS, R. Simple slide and tube test for infectious mononucleosis. *Am J Clin Path.* 6: 546, 1936.
- <sup>c</sup> BERNSTEIN, A. Diagnostic importance of the heterophile antibody test in leukemia. *J. Clin. Investigation* 13: 677, 1934.
- <sup>d</sup> BUTT, E. M., AND FOORD, A. G. The heterophile antibody reaction in the diagnosis of infectious mononucleosis. *J. Lab. & Clin. Med.* 20: 583, 1935.
- <sup>e</sup> SCHIFF, F. Heterogeneous hemagglutinins in man following therapeutic injection of immune sera produced in rabbits. *J. Immunol.* 33: 305, 1937.
- <sup>f</sup> BERNSTEIN, A. Antibody responses in infectious mononucleosis. *J. Clin. Investigation* 13: 419, 1934.
- <sup>g</sup> BERNSTEIN, S. Heterophile antibody reaction caused by bacterial infection. *Ann. Int. Med.* 16: 474, 1942.
- <sup>h</sup> KAUFMAN, R. E. Heterophile antibody reaction in infectious mononucleosis. *Ann. Int. Med.* 21: 230, 1944.
- <sup>i</sup> STUART, C. A., GRIFFIN, A. M., MACDONALD, F., AND ANDERSON, E. G. B. Nature of the antibodies for sheep cells in infectious mononucleosis. *Proc. Soc. Exper. Biol. & Med.* 34: 209, 1936.

- <sup>1</sup> STUART, C. A., GRIFFIN, A. M., WHEELER, K. M. AND BATTEY, S. A thermostable antigen in beef cells  
Proc Soc Exper Biol & Med 34 212, 1936
- <sup>2</sup> WIENER, A. S. A new test (blocking test) for Rh sensitization Proc Soc Exper Biol & Med 56  
173, 1944
- <sup>3</sup> RACE, R. R. An incomplete antibody in human serum Nature 153 771, 1944
- <sup>4</sup> DIAMOND, L. K., AND ABELSON, N. M. The demonstration of anti Rh agglutinins. An accurate and  
rapid slide test J A M A 30 204, 1945
- <sup>5</sup> LEVINE, P., AND GILMORE, E. L. The first stage of antibody reaction in infectious mononucleosis  
Science 101 410, 1945
- <sup>6</sup> MOLONEY, WILLIAM C. Unpublished data
- <sup>7</sup> HANOER, F. M. Serological differentiation of obstructive from hepatogenous jaundice by flocculation  
of cephalin-cholesterol emulsions J Clin Investigation 18 261-269, 1939
- <sup>8</sup> MACLAOEN, N. F. Thymol turbidity test. New indicator of liver function. Preliminary report Nature  
154 670, 1944
- <sup>9</sup> KUNKEL, G. H. Estimation of alterations of serum gamma globulin by a turbidimetric technique  
Proc Soc Exper Biol & Med 66 217, 1947

# THE PARALLEL EFFECTS OF MAGNESIUM ON THE COMPLEMENTARY AND COAGULATIVE ACTIVITIES OF BLOOD SERUM

By FRANK MALTANER, PH D, AND JOSÉ O DE ALMEIDA, M.D

THE CLOSE relationship between the role of calcium in the clotting and complement activities of serum has already been shown.<sup>1</sup> More recent studies have demonstrated the effect of magnesium in the hemolytic activity of complement<sup>2</sup> and in the clotting reaction.<sup>3</sup> Since it is well known that magnesium salts do not replace calcium in the clotting process, this work suggested that the part of magnesium be further explored. It has been found that the effect of magnesium ions in both phenomena is parallel, either alone or in the presence of such antagonists as human serum and disodium phosphate. The practical importance of ionized magnesium salts in complement-fixation tests has also been emphasized by other investigators.<sup>2</sup> Experiments were therefore made to determine whether addition of these salts affects the quantitatively standardized complement-fixation procedure developed in this laboratory.

## MATERIALS AND METHODS

Complement was obtained from frozen pools of guinea pig serum used in routine complement fixation tests.<sup>4</sup>

Inactivated human serum was a pool of sera inactivated at 56 C. for one half hour prior to use.

Disodium phosphate was prepared by diluting 1/10th molar solutions with physiologic salt solution.

Magnesium chloride and calcium chloride were similarly prepared from molar solutions of these salts.

Cephalin was phosphatidyl serine prepared by the method of Folch,<sup>5</sup> further purified by reprecipitation from hot methyl alcohol,<sup>6</sup> and dissolved in petroleum ether. It was suspended in distilled water from the dried state as 0.1 per cent solution, was dialyzed overnight, made isotonic, and, diluted 1:10 with physiologic salt solution.

Dioxalated plasma,<sup>7</sup> used for titration of prothrombin activity, was prepared from cell free 0.1 per cent oxalated plasma obtained by the carotid bleeding of guinea pigs using paraffined canulae and tubes chilled in ice. Nine volumes of blood were collected in one volume of 1 per cent sodium oxalate dissolved in 0.5 per cent sodium chloride. After removal of blood cells at low speed the plasma was transferred to paraffined tubes and the platelets removed as completely as possible by high speed centrifugation for one to two hours in the refrigerated centrifuge. The horizontal position in the centrifuge should be used and the time required depends on the speed available. A plasma that clots in ten minutes or longer in glass tubes and after optimum recalcification may be used but much more stable plasma is obtainable by these procedures. The dioxalated plasma was prepared by diluting oxalated plasma with four volumes of 0.1 per cent sodium oxalate in physiologic salt solution.

The hemolytic system was prepared from washed 5 per cent sheep cells and antisheep cell amboceptor.<sup>4</sup>

## Clotting Technic

The quantity of complement selected for use in clotting tests was one unit as employed in the quantitatively standardized complement-fixation test for syphilis, 1 c, the amount required for 50 per cent hemolysis of a standard dose of maximally sensitized sheep cells in fifteen minutes in a water bath at 37 C. Amounts of calcium

From the Division of Laboratories and Research, New York State Department of Health, Albany, N. Y.  
Presented at the meeting of the American Association of Immunologists, March 17, 1948, Atlantic City, New Jersey.

chloride and cephalin were used that, when incubated for six minutes in preliminary titration with one unit of complement in a total volume made up to 0.6 ml with physiologic salt solution, clotted 0.1 ml of added dioxalated plasma in ten minutes. In the experiments recorded below, this corresponded to 0.1 ml of  $M/400$  calcium chloride and 0.1 ml of 0.01 per cent phosphatidyl serine.

In the tests of the inhibiting effect of inactivated human serum and disodium phosphate and of the enhancing action of magnesium chloride, varying amounts of these reagents were pipetted into the test tubes followed by complement, calcium chloride, cephalin, and physiologic salt solution to a volume of 0.6 ml. The mixture was incubated in the water bath at 37 C for six minutes and 0.1 ml of dioxalated plasma was then added. The clotting time was recorded in minutes.

#### *Technic of Complement Titration*

Two methods of complement titration were employed, one based on the time and the other on the amount required for 50 per cent hemolysis at constant time. Similar results were obtained with both methods but only the former is described since it provides a more convenient comparison with the results of the clotting tests.

Determinations of time of hemolysis were made in a total volume of 2.0 ml in the calibrated tubes of a Coleman Junior spectrophotometer at wave length 580  $\mu$ . The T per cent transmission reading corresponding to 50 per cent hemolysis was determined by measurement of color standards prepared from known proportions of hemolyzed and unhemolyzed cells and an amount of inactivated complement similar to that used in the tests. The readings were made with the cells in suspension. In the preliminary titration, complement was used in amounts of 0.4, 0.2, 0.15, and 0.1 ml of a 1:25 dilution. Volumes were equalized with physiologic salt solution to 1.6 ml before the addition of 0.4 ml of sensitized sheep cells. Incubation was at 37 C in the water bath and the time required for 50 per cent hemolysis was recorded. An amount of complement which required twelve minutes for 50 per cent hemolysis was used in the following experiments. The different amounts of inactivated serum, disodium phosphate, and magnesium chloride were added to this unit quantity of complement, the volumes made up to 1.6 ml with physiologic salt solution, and 0.4 ml of sensitized cells added. Readings were made at frequent intervals during incubation and the time required for 50 per cent hemolysis recorded.<sup>8</sup>

#### *Technic of Quantitative Titration of Syphilitic Sera by Complement Fixation*

Method I<sup>14</sup> was employed but in one set of tests physiologic salt solution containing 12 micrograms of magnesium per ml for diluting antigen, amboceptor, and complement, and for equalizing volumes in different tubes of titrations were used.

### RESULTS

*The effect of magnesium chloride on the inhibition of prothrombin activation by inactivated human serum and by disodium phosphate.* Figure 1, curve 1 shows the effect of magnesium chloride on the prothrombin activation of complement. The clotting

time was reduced from ten to two minutes by the addition of 0.1 ml of a  $M/800$  solution of magnesium chloride. Curve 2 shows the effect of magnesium chloride in the presence of an inhibiting dose of inactivated human serum which without magnesium chloride gave a clotting time of fourteen minutes. The inhibitory effect was neutralized by magnesium chloride, maximum activation resulting with 0.18 ml in three minutes. Curves 3 and 4 show the effect of magnesium chloride on inhibition by disodium phosphate. One-tenth of a milliliter of a  $M/100$  solution of the phosphate showed a clotting time of fourteen minutes. This was reduced progressively with increasing quantities of magnesium chloride to two minutes. With double the amount of disodium phosphate, prothrombin activation of one hemolytic unit of complement was completely inhibited but twice the amount of added magnesium chloride completely neutralized the effect of this dose. These results suggested an equivalent relationship in the effect of these two salts.

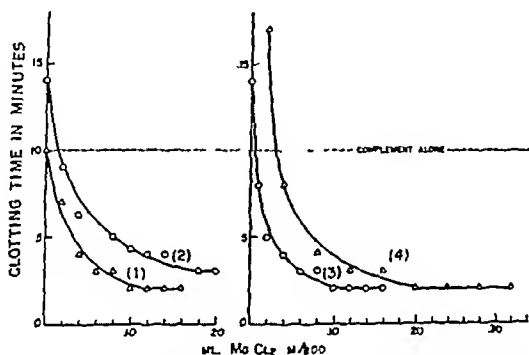


FIG. 1—Curves 1 and 2. Effect of inactivated human serum on the enhancing action of magnesium chloride in the prothrombin activation of complement.

Curves 3 and 4. The effect of disodium phosphate ( $\text{Na}_2\text{HPO}_4$ ) on the enhancing action of magnesium chloride in the prothrombin activation of complement.

*Effect of magnesium chloride on the inhibition of the hemolytic activity of complement by inactivated human serum and disodium phosphate (figures 2 and 3).* The time required for 50 per cent hemolysis in the absence of serum and magnesium chloride was 12.2 minutes. This was increased in the presence of increasing amounts of serum to 18.8 minutes with 0.2 ml. When this dose of inactivated human serum was tested with magnesium chloride in varying amounts, the inhibiting effect of the serum was neutralized by approximately 0.35 ml of a  $M/100$  solution, and 0.6 ml resulted in a further enhancement as indicated by the reduction in time for 50 per cent hemolysis to 11 minutes. Similarly, in tests with the same quantity of complement and varying quantities of  $M/20$  sodium phosphate, slight activation was observed with 0.1 ml and inhibition with larger amounts. Five tenths of a milliliter required 22.5 minutes for 50 per cent hemolysis as shown in the first part of figure 3. As shown in the second part of figure 3, when varying quantities of magnesium chloride were used with this dose of disodium phosphate, 0.25 ml of an  $M/500$  solution of magnesium chloride completely neutralized the inhibitory

effect of the phosphate and further enhancement resulted with increasing quantities up to 0.6 ml

*Effect of magnesium chloride on the titer of syphilitic sera as determined by the quantitatively standardized complement-fixation procedure (table 1)* Under the conditions

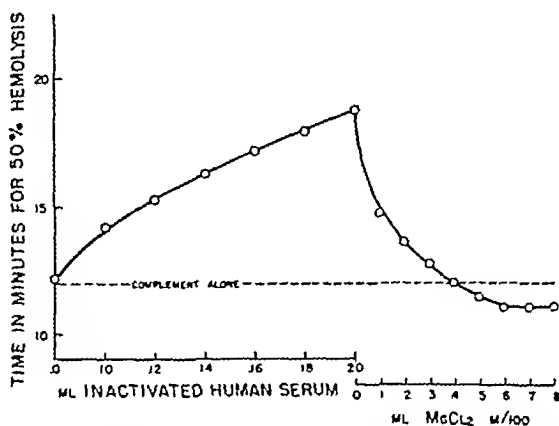


FIG. 2.—The inhibiting effect of inactivated human serum on hemolysis by complement. The effect of inactivated human serum on the enhancing action of magnesium chloride for hemolysis by complement.

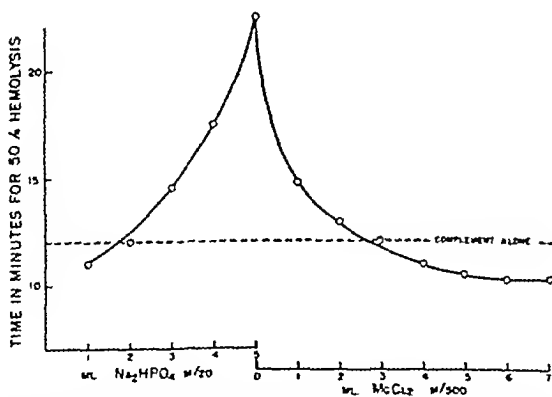


FIG. 3.—The inhibiting effect of disodium phosphate on hemolysis by complement. The effect of disodium phosphate on the enhancing action of magnesium chloride for hemolysis by complement.

employed in the test a linear relationship is observed between the amount of complement required for 50 per cent hemolysis and the amount of serum tested, the total change in complement activity or total fixation of complement is determined by linear extrapolation. The unit value as determined by titration of complement alone does not represent the unit value under the conditions of the

test, i e., in the presence of serum and antigen. The cardiolipin antigen used in these tests has, in itself, little or no effect upon complement. The serum of the test, however, has a variable effect. Therefore the titer is taken as the ratio of the total change to the change resulting from incubation with serum alone. In table 1 it may be seen that the total reaction of serum plus antigen observed with 6 different sera in tests with and without magnesium, varied markedly but the titers when expressed as the ratio  $\frac{\text{serum} + \text{antigen}}{\text{serum alone}}$  were essentially the same.

TABLE 1—The Effect of  $Mg^{++}$  Treated Complement in the Quantitative Complement Fixation Test for Syphilis

Serum no	Complement unit		Reaction				Titer $\frac{\text{Serum} + \text{antigen}}{\text{serum alone}}$	
	Saline	$MgCl_2$ Saline	Serum + antigen		Serum alone		Saline	$MgCl_2$ saline
			Saline	$MgCl_2$ saline	Saline	$MgCl_2$ saline		
174261	0 0016	0 00096	75	111	1 19	1 78	60	61
174262	0 0015	0 0007	74	99	1 32	1 54	56	64
174263	0 0016	0 00096	72	126	1 12	1 78	64	70
184442	0 0015	0 00088	323	434	1 39	2 00	132	217
192572	0 0015	0 00088	477	751	1 78	3 00	263	250
197004	0 0015	0 0010	332	416	1 60	2 00	207	208

### DISCUSSION

The results confirm those of previous investigators<sup>2</sup> in showing the enhancement of the hemolytic effect of complement by magnesium. They demonstrate also a parallel effect of magnesium on the coagulative activity of serum. In both cases the influence of magnesium is inhibited by serum and by disodium phosphate. It has been suggested<sup>2</sup> that the greater effect of magnesium ions over calcium or other cations on the hemolytic activity of complement, implies its greater importance in this phenomenon. Indeed the explanation has been offered that calcium may act by displacement of magnesium from a complex. It should be borne in mind that the opposite may also be true, namely, that the enhancing effect of magnesium ions is due to a sparing of calcium ions from the serum phosphates. The fact that the addition of ionized calcium salts to complement does not increase significantly its hemolytic activity appears to render this explanation unlikely, but it is indeterminate whether or not the addition of amounts of  $Ca^{++}$  equivalent to those that might be liberated as a result of the sparing action of  $Mg^{++}$  would increase the calcium ion concentration of serum. On the other hand, the idea of a sparing effect on calcium seems logical in relation to the clotting phenomenon, in which ionized magnesium salts appear to be inactive in the absence of calcium. The parallel behavior of magnesium in enhancing the clotting and complement activities of serum, and parallel behavior of disodium phosphate and of serum in antagonizing this effect suggest a common cause, whatever it may prove to be.

The use of magnesium chloride may introduce error into complement fixation tests when the effect on the reaction of syphilitic serum and antigen is not considered in relation to the effect on the reaction of serum alone.

## SUMMARY

Magnesium chloride enhances the coagulation and complement activities of blood serum in parallel degree. These enhancing effects are inhibited by inactivated serum or by disodium phosphate.

Distortion in the results of complement-fixation tests occurs with the addition of ionized magnesium salts to the system. This is due to the antagonistic effect of the inactivated test serum. In the quantitatively standardized test, however, where the titer is expressed as the ratio of the reaction of serum and antigen to that of serum alone, the findings are the same with or without added magnesium salts.

## REFERENCES

- <sup>1</sup> MALTANER, F., AND JOHNSTON, E. Observations upon the coagulation phenomenon. *J Immunol* 6 349-354, 1921, WADSWORTH, A. MALTANER, F. AND MALTANER, E. A study of the coagulation of the blood: the chemical reactions underlying the process. *Am J Physiol* 80 502-521, 1927, IDEM. Further studies of the chemical reactions underlying the coagulation of the blood: the activity of cephalin. *Am J Physiol* 97 74-81, 1931, IDEM. Studies on the coagulation of the blood. A. *Am Phys*, Tr 46 296, 1931, IDEM. Studies on the activity of cephalin as it relates to the coagulative and complementary properties of the blood. *J Immunol* 30 417-444, 1936, IDEM. The inhibition of complementary activity by anticoagulants. *J Immunol* 33 297-303, 1937, MALTANER, F. The significance of thromboplastic activity of antigens used in complement fixation tests. *Proc. Soc. Exper. Biol. & Med.* 62 302-304, 1946.
- <sup>2</sup> MAYER, M. M., OSLER, A. G., BIER, O. G., AND HEIDELBERGER, M. The activating effect of magnesium and other cations on the hemolytic function of complement. *J. Exper. Med.* 84 535-548, 1946.
- <sup>3</sup> MALTANER, F. The enhancing effect of  $Mg^{++}$  on the clotting activity of  $Ca^{++}$ . *Proc. Soc. Exper. Biol. & Med.* 67 301-303, 1948.
- <sup>4</sup> WADSWORTH, A. Standard Methods of the Division of Laboratories and Research of the New York State Department of Health 3d ed. Baltimore, Williams & Wilkins 1947 990 p (2) p 440-443.
- <sup>5</sup> FOLCH, J. Brain cephalin: a mixture of phosphatides. *J. Biol. Chem.* 146 35, 1942.
- <sup>6</sup> MALTANER, F. A note on the preparation of cephalin. *J. Am. Chem. Soc.* 53 4019, 1931.
- <sup>7</sup> BORDET, J., AND DELANOE, L. La coagulation du sang et la genèse de la thrombine. *Ann. l'Institut Pasteur* 26 657-674, 1912.
- <sup>8</sup> ALMEIDA, J. O. Equivalência entre o tempo da hemólise e a concentração do complemento. *Rev. Paulista Med.* 30 44-45, 1947.



## THE ABSENCE OF THE HETEROPHILE REACTION IN THE SPINAL FLUID IN INFECTIOUS MONONUCLEOSIS

By COMMANDER HAROLD A. LYONS (MC) USN, AND LT (JG) J. GRANT HARRISON  
(MC) USN

THE DEMONSTRATION in 1932 by Paul and Bunnell<sup>1</sup> that the blood serum of patients with the sporadic form of infectious mononucleosis contained antibodies against sheep erythrocytes in concentrations far above a normal titer was an important advance in the study and diagnosis of this disease. It gave a good test for the recognition of the individual case, allowed the linking of the sporadic and the epidemic forms of the disease<sup>2</sup> and permitted the establishment of certain epidemic fevers as infectious mononucleosis.<sup>3</sup> The test has been reported to be positive in 92 per cent of the sporadic cases,<sup>4</sup> and is consistently negative when performed in many diseases.<sup>5-7</sup> Recently, Gutner and Fisher<sup>8</sup> have described a case of chronic melioidosis with a persistently high heterophile titer (None of the differential absorption tests described below were done.) Even in those cases in which horse serum was previously administered, the reaction can be differentiated from infectious mononucleosis by the appropriate methods.<sup>9, 10</sup> Conditions producing Forssman type antibodies (such as *E. coli* bacteremia, the use of parenteral liver extract, etc.) can be distinguished by the fact that the antibodies are not absorbed by ox cells.<sup>11-13</sup> In infectious mononucleosis, the antibodies are absorbed by ox cells but not by guinea pig kidney, whereas, in normal serum the antibodies are absorbed by guinea pig kidney but not by ox cells. These differential absorption tests distinguish the various types of heterophile antibodies.<sup>10, 14</sup>

Formerly, it was thought that the antibodies of infectious mononucleosis were of the Forssman type, but now, it is definitely known that they are not. The differential absorption tests described above distinguish the heterophile antibodies occurring in various conditions from those of infectious mononucleosis. Warren<sup>14</sup> believes that the two types are interrelated, but this view is not widely held.

The spinal fluid has on occasion shown abnormalities. The pressure may be moderately elevated. Cell count increase is variable, ranging from twenty-five to three hundred cells. Lymphocytes usually predominate.<sup>15, 16, 22</sup> This is not at all uncommon, as was found in our cases. The spinal fluid sugar, and the chloride content are usually normal. The protein, however, may be increased and the Pandy Test strongly positive. Usually, the increase in the protein is out of proportion to the cell count.

It is interesting to note that although spinal fluid abnormalities have been studied in infectious mononucleosis,<sup>17-20</sup> little attention has been directed as to whether or not the heterophile antibody is present. Slade<sup>21</sup> and Landes, Reich and Perlow,<sup>22</sup> respectively, found a negative spinal heterophile in one case. Ab-

From the Medical Service, U. S. Naval Hospital, St. Albans, New York.

The opinions expressed by the writers are their own and are not to be taken as reflecting the opinions or policies of the Naval Service at large.

normal spinal fluid findings of pleocytosis and increased protein, however, were present

We have examined the spinal fluids in 20 cases with positive heterophile agglutinations in the serum. These findings are summarized in table 1.

The spinal fluid abnormalities that occur are similar to those reported in the literature by Thelander and Shaw<sup>23</sup> and Shafer and Weir,<sup>24</sup> with pleocytosis and

TABLE 1—*Blood Serum and Spinal Fluid Findings in Infectious Mononucleosis*

Blood Serum		Spinal fluid			
Heterophile Agglutination		Cell count	Protein	Sugar	Chlorides
Case	Titer				
1 J. B.	1:112	0	50.0	55.6	—
2 G. H.	1:112	—	64.6	—	—
3 L. G.	1:488	9(5L, 4S)	37.2	41.2	73.0
4 M. M.	1:28* 1:1792†	—	23.9	67.5	—
5 T. S.	1:1340	—	29.3	—	—
6 D. J.	1:28* 1:112†	3(3L)	—	—	—
7 C. H.	1:1340	0	41.5	—	—
8 R. H.	1:1792	16(16L)	27.3	47.5	81.0
9 A. M.	1:1792	13(9L, 4S)	—	—	—
10 A. S.	1:1992	0	—	—	—
11 L. S.	1:876* 1:992†	3(3L)	30.6	69.2	—
12 R. E.	1:14* 1:448†	—	—	—	—
13 R. M.	1:896	—	—	—	—
14 W. O. H.	1:14* 1:1792†	—	—	—	—
15 P. P.	1:14* 1:448†	—	—	—	—
16 D. K.	1:448	—	—	—	—
17 D. J.	1:56	—	—	—	—
18 P. D.	1:448	0	—	—	—
19 R. N.	1:1340†	15(15L)	68.0	45	71.0
20 S. K.	1:4970	0	30.0	53	61.5

L = Lymphocyte

S = Segmented cell.

— = Not done.

1:56 positive dilution was considered a positive test

Heterophile titer was negative in all instances

\* 1st week of illness

† 2nd week of illness

† Also had in the right lung a solitary lesion of coccidioidomycosis with a positive complement fixation and precipitin titers for coccidioidomycosis.

increased protein as the outstanding features. The cases described in this paper were free of any central nervous system manifestations. As will be seen from the table, there is no correlation between the serum heterophile agglutination titer and the other spinal fluid changes. Each case revealed an absence of heterophile agglutinins in the spinal fluid.

Landes<sup>1</sup> commented on the lack of reports of spinal fluid examinations for heterophile agglutinins. As noted above, he and Slade demonstrated a lack of

heterophile agglutinins in the presence of other abnormalities. This finding has been confirmed in the 20 cases in the table, and if their 2 cases are included, it is true for twenty-two cases.

Each case had a diagnostic titer of heterophile agglutinins in the blood serum, although agglutinins were lacking in the spinal fluid, even when other abnormalities were present. Even in the presence of spinal fluid abnormalities, clinical evidence of central nervous system symptoms or signs were lacking, but this certainly does not preclude central nervous system involvement by the disease. The converse also is true of infectious mononucleosis with cerebral signs and symptoms. Such cases may yield normal spinal fluid studies<sup>27</sup> or may parallel the neurologic involvement.<sup>28</sup>

The central nervous symptoms that occur are identified by various authors<sup>16-17, 19-24</sup> as meningitis, serous meningitis, lymphocytic meningitis, metastatic encephalomyelitis, encephaloneuritis, encephalomyelitis, neuronitis, and Guillain Barré syndrome. These various designations merely indicate the varied ways that the central nervous system is involved in infectious mononucleosis. Berce<sup>24</sup> has demonstrated with electroencephalograms that encephalitic foci are present with or without demonstrable cerebral symptoms.

The abnormal spinal fluid findings may thus be associated with various types of involvement of the nervous system, or with none. Whatever the heterophile antibody is in infectious mononucleosis, it does not appear to pass from the blood serum into the cerebrospinal fluid. Likewise, whatever the mechanism is in infectious mononucleosis that produces the not infrequently positive Wassermann reaction in the serum, it does not affect the spinal fluid, for in all the studies this reaction has been consistently negative.<sup>25, 27</sup> In what manner the other abnormal spinal fluid changes are brought about is not known. They may be due to the virus which is the probable etiologic agent of the disease or to a possible allergic reaction.<sup>28</sup> The abnormal number of lymphocytes in the spinal fluid may simply be a reflection on blood lymphocytosis, or the lymphocytes may be squeezed out from the perivascular spaces.

Jervis,<sup>29</sup> in keeping with the allergic concept, produced an acute disseminated encephalomyelitis by injection of Forssman antibodies into the carotid arteries of guinea pigs. He believed the Forssman antibody passed through an impaired blood brain barrier. However, in the experiments of Jervis, the guinea pig tissue, including the brain, probably contains Forssman antigen with which the injected antibody might well react. Human tissues do not contain Forssman antigen.

Recent electrophoretic studies of the serum proteins throw some light on the subject of antigen-antibody reaction, but have not as yet been extensively studied in the spinal fluid.<sup>30, 31</sup> It has been shown that the beta and gamma globulin serum proteins are elevated, and that the heterophile reaction, like all antibody reactions, is due to this elevation.

Because of the protein manifestations of infectious mononucleosis, and the delayed development of the heterophile reaction or the lack of its presence in the serum, abnormal spinal fluid findings might be misleading. The fact that a heterophile antibody does not occur in the spinal fluid reveals that this cannot aid in the diagnosis.

## SUMMARY

The spinal fluid was studied in twenty cases of infectious mononucleosis proved by clinical picture, blood studies and serological examination. It may be concluded that in acute cases of infectious mononucleosis, the heterophile antibody does not pass into the spinal fluid, but that there may be an increase in cell count, particularly lymphocytes, and in protein content, which is not necessarily proportional to the cell count elevation. These findings have no correlation with central nervous system signs or symptoms.

## REFERENCES

- <sup>1</sup> PAUL J. L., AND BUNNELL, W. W. The presence of heterophile antibodies in infectious mononucleosis. *Am. J. M. Sc.* 183 90 1932.
- <sup>2</sup> NOLAN R. A. Report of so-called epidemic of glandular fever (infectious mononucleosis). *U. S. Nav. Med. Bull.* 33 479, 1935.
- <sup>3</sup> USABINA D. Hanguntzin—Deichersche Reaktion bei endemischem Fieber in Süd Japan. *Nagoya J. Med. Sc.* 11 79 1937.
- <sup>4</sup> BERNSTEIN A. Infectious mononucleosis. *Medicine* 19 85, 1940.
- <sup>5</sup> DE VRIES S. I. The icteric form of glandular fever. *Acta Med. Scandinav.* 95 552, 1938.
- <sup>6</sup> KAUFMAN, R. E. Heterophile antibody reaction in infectious mononucleosis. *Ann. Int. Med.* 21 230 1944.
- <sup>7</sup> KENT C. F. False positive Paul Bunnell (heterophile) reaction? *Am. J. Clin. Path.* 10 576 1940.
- <sup>8</sup> GUTNER LEONARD B., AND FISHER MYRON W. Chronic melioidosis. Discussion, case report and special studies. *Ann. Int. Med.* 28 6 1157-1186 1948.
- <sup>9</sup> DAVIDSOHN J. Serologic diagnosis of infectious mononucleosis. *J. A. M. A.* 108 289, 1937.
- <sup>10</sup> — Test for Infectious Mononucleosis. *Am. J. Clin. Path.* 8 56, 1938.
- <sup>11</sup> BORNSTEIN, S. Heterophile antibody reaction caused by bacterial infection. *Ann. Int. Med.* 16 472, 1942.
- <sup>12</sup> STUART, C. A., FULTON, M. ASH, R. P. AND GREGORY K. K. The relations between certain heterophile antibodies and antigens. *J. Infect. Dis.* 59 65 1936.
- <sup>13</sup> DAVIDSOHN, J. AND WALKER P. The nature of the heterophile antibodies in infectious mononucleosis. *Am. J. Clin. Path.* 5 455 1935.
- <sup>14</sup> WARREN, E. W. Observations on infectious mononucleosis. *Am. J. M. Sc.* 201 483 1941.
- <sup>15</sup> ZIEGLEN, E. E. Infectious mononucleosis. Fatal case with autopsy. *Arch. Path.* 37 196 1944.
- <sup>16</sup> ZOHMAN, B. L., AND SILVERMAN E. G. Infectious mononucleosis and encephalomyelitis. *Ann. Int. Med.* 16 1233 1942.
- <sup>17</sup> HABER W. Reitling zur Meningitis Serosa bei Pfeiffer's likem Drüsenfieber. *Schweiz. Med. Wchnschr.* 68 892, 1938.
- <sup>18</sup> EPSTEIN S. H. AND DAMESHEK W. Involvement of the central nervous system in a case of glandular fever. *New England J. Med.* 205 1238 1931.
- <sup>19</sup> SUCHER A. AND SCHWARZ E. Lymphatische Reaktion und Encephalomenigitis. *Wien. Klin. Wchnschr.* 49 1417, 1936.
- <sup>20</sup> GSELL, O. Meningitis serosa bei Pfeifferschen Drüsenfieber (Mononucleosis Infectiosa). *Deutsch. Med. Wchnschr.* 63 1759 1937.
- <sup>21</sup> SLADE JOHN DE R. Involvement of the central nervous system in infectious mononucleosis. *New England J. Med.* 234 23, 753-57 1946.
- <sup>22</sup> LANDIS, R. REICH J. P. AND PERLOW S. Central nervous system manifestations of infectious mononucleosis. Report of a case. *J. A. M. A.* 116 2482-2484 1941.
- <sup>23</sup> THELANDER H. E. AND SHAW E. B. Infectious mononucleosis with special reference to cerebral complications. *Am. J. Dis. Child.* 61 1131-1145 1941.
- <sup>24</sup> SHAPER J., AND WEIR J. C. R. Meningeal reaction in case of glandular fever. *Brit. M. J.* 1 118 1943.
- <sup>25</sup> GOLDSTEIN J. D., as quoted in BERNSTEIN A. Infectious mononucleosis. *Medicine* 19 85, 1940.
- <sup>26</sup> BERGEL, NICHOLAS A. The electro-encephalogram in cerebral complications of infectious mononucleosis. *J. Nerv. & Ment. Dis.* 107 6, 537-544 1945.

- <sup>27</sup> LOHE, H , AND ROSENFELD H   Über Monozytenangina mit anschliessendem vorübergehend seropositiven Erythema nodosum, zugleich ein Beitrag zur Differentialdiagnose zwischen luetischer und nichtluetischer Angina. *Sermat. Ztschr* 53 373, 1928
- <sup>28</sup> STEVENSON, L. D , AND ALVARD, E. C , Jr   Allergy in the Nervous System *Am. J Med* 3. 614-619 Nov 1944
- <sup>29</sup> JERVIS, G   Forsman's Carotid Syndrome, a contribution to the study of anaphylactic changes in the nervous system from the standpoint of pathology *Arch Path* 35 560, 1943
- <sup>30</sup> COHEN, P P , AND THOMPSON, B S   Mechanism of the thymol turbidity test *J Lab & Clin Med* 32. 475-481, 1947
- <sup>31</sup> LONGSWORTH, L. G   Recent advances in the study of proteins by electrophoresis *Chem Rev* 31 323 1942-
- <sup>32</sup> EVANS, ALFRED S   Liver involvement in infectious mononucleosis *J Clin Investigation* 27 1 106 1948

# A FACTOR IN SERUM WHICH ACCELERATES THE CONVERSION OF PROTHROMBIN TO THROMBIN

## II ITS EVOLUTION WITH SPECIAL REFERENCE TO THE INFLUENCE OF CONDITIONS WHICH AFFECT BLOOD COAGULATION

By BENJAMIN ALEXANDER, M D , ANDRE DE VRIES, M D ,\* AND  
ROBERT GOLDSTEIN, M D

*With the technical assistance of EUNICE ADDELSON and ELAINE PROMISEL*

IN A PREVIOUS communication,<sup>1</sup> a serum factor was described which accelerates the conversion of plasma prothrombin to thrombin by thromboplastin plus calcium, and a method for its determination was reported. This agent, labelled serum prothrombin conversion accelerator (SPCA), is distinct from thrombin, thromboplastin and labile factor. Insufficient data are available to establish the identity or nonidentity of this factor with serum Ac globulin of Ware et al.<sup>2</sup> or Factor VI of Owren,<sup>3</sup> substances with similar physiologic properties.

This paper presents data concerning the evolution of SPCA in human subjects under certain conditions which affect blood coagulation. Similar observations in various hemorrhagic disorders are reported elsewhere in this issue.<sup>4</sup>

### METHOD

SPCA was determined by a method based upon the effect of the admixture of serum on the prothrombin time of normal plasma.<sup>1</sup> The activity of SPCA is expressed as the enhancement, in per cent, of the prothrombin activity of a serum plasma mixture over and above the algebraic sum of the prothrombin activities of each component. Coagulation time of whole blood was determined by a modification of the Lee and White method.<sup>5</sup>

### RESULTS

*Evolution of SPCA following Coagulation* In previous studies, SPCA was demonstrated in serum obtained from normal blood 1 hour after coagulation.<sup>1</sup> The amount of SPCA which evolves at various intervals after clotting is shown in table 1. Immediately after coagulation, SPCA is low, whereas, as has also been shown by other investigators,<sup>6</sup> serum prothrombin is high. Within 15 minutes, SPCA increases concomitant with a decrease in serum prothrombin activity. During the next 45 minutes, some prothrombin activity tends to reappear in the serum, and SPCA activity tends to decrease somewhat.

*Normal Variation in SPCA and Serum Prothrombin* The SPCA in 95 normal subjects one hour after blood coagulation varied between 43 and 271 (fig. 1) with a mean of 99.4. The prothrombin activities of the same sera ranged between 0 and 32 per cent.

From the Medical Research Laboratories, Beth Israel Hospital and the Department of Medicine, Harvard Medical School, Boston.

This study supported by a grant from the Commonwealth Fund.

\* Aided by a fellowship from the Rothschild Hadassah University Hospital, Jerusalem.

(fig. 2) with a mean of 6.4. No correlation was evident between the amount of SPCA and the serum prothrombin-activity, or the difference in prothrombin ac

TABLE 1—SPCA Activity at Various Intervals after Coagulation

Interval after clot	SPCA	Serum proth. activity
min	per cent	per cent*
<5	19	42
15	95	4
30	78	11
60	55	19
120	65	16
180	77	15

\* The prothrombin activity of normal plasma is considered to be 100 per cent.

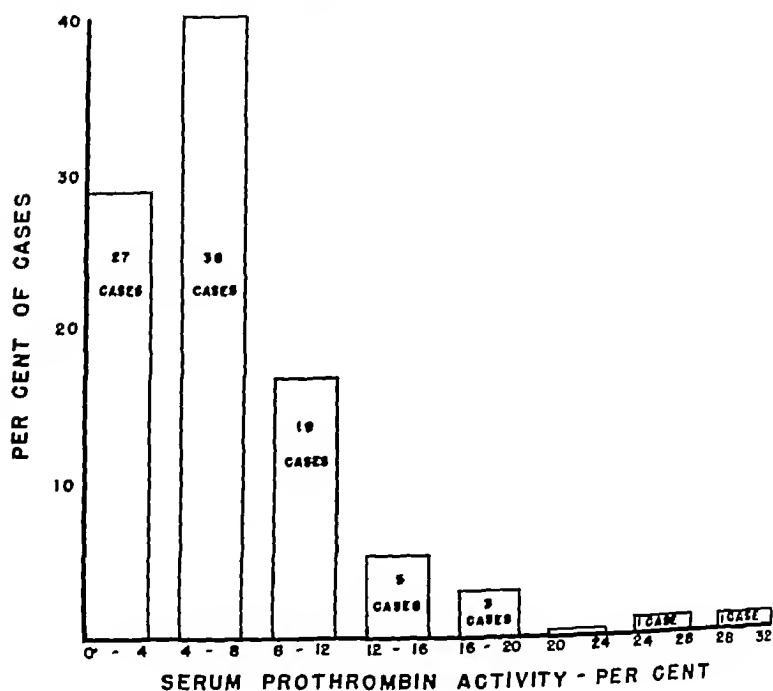


FIG. 1

tivity of the plasmas and their respective sera. Nor was there any apparent relation between the clotting time in glass and SPCA or serum prothrombin activity.

*Effect of Accelerating Coagulation.* It is well known that agitating freshly drawn blood accelerates coagulation. This procedure also accelerates SPCA evolution, increases the amount of it evolved and decreases residual serum prothrombin

activity (table 2) Defibrination of freshly drawn blood by vigorous shaking yields serum which is very rich in SPCA and practically free of prothrombin activity. The addition of rabbit brain thromboplastin extracts (prepared as for prothrombin determinations from Difco thromboplastin) to blood also accelerates its

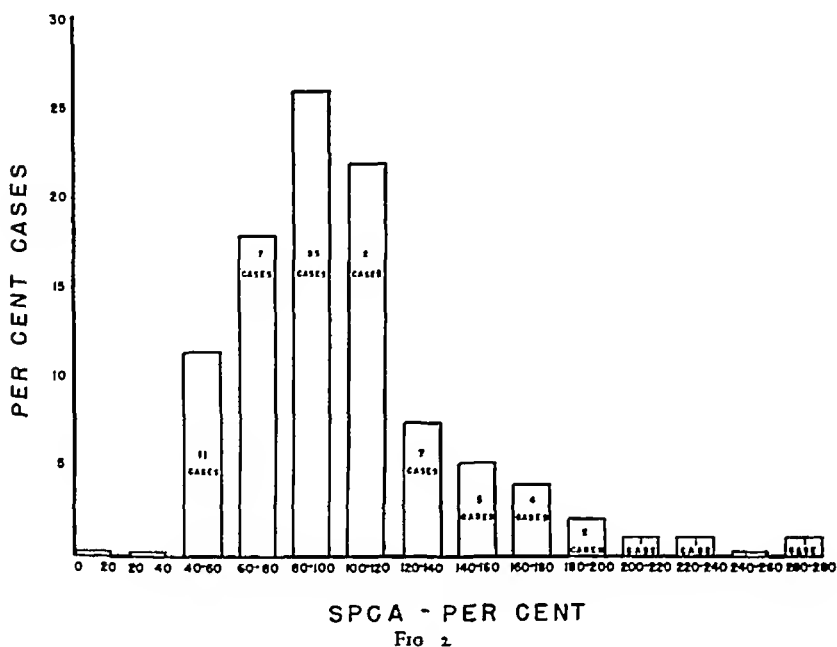


TABLE 2.—Effect of Agitation of Blood on Evolution of SPCA

Experiment	Remarks	SPCA (per cent)		Serum prothrombin activity (per cent)	
		Non agit	Agit	Non agit	Agit.
I	Centrifuged and oxalated immed. after coagulation	19	76	42	5
II	Centrifuged and oxalated immed after coagulation	27	80	51	14
III	The agitated sample was defibrinated by shaking for 8 minutes, which was the clotting time of the non agitated sample	44	100	56	<3

\* The prothrombin activity of normal plasma is considered to be 100 per cent.

coagulation, increases in most instances the amount of SPCA\* and at the same time renders the serum practically devoid of prothrombin activity (table 3) The question whether this effect of thromboplastin supplements is intimately related to

\* Strangely enough the addition of rabbit brain thromboplastin to freshly drawn dog blood results in decreased SPCA in contrast to the effect on human blood



actual clotting was studied by adding thromboplastin to serum. The addition of thromboplastin to *nonoxalated* serum increases the amount of SPCA activity in contrast to what obtains with *oxalated* serum. The serum prothrombin activity was unaffected in 1 experiment and decreased slightly in another, but was always demonstrable whereas the serum from blood clotted with thromboplastin supplements was always devoid of prothrombin activity.

SPCA has been shown to be distinct from thromboplastin.<sup>1</sup> Nevertheless it is conceivable, in view of evidence that thromboplastin is not consumed during coagulation,<sup>7</sup> that the enhancement in SPCA induced by additions of thromboplastin might be related to unconsumed thromboplastin remaining in the serum. Experiments, designed to explore this possibility, revealed that thromboplastin, added to oxalated serum, in proportions comparable to those added to blood not only failed to increase the SPCA activity but in some instances decreased it.\*

TABLE 3—Effect of Thromboplastin Supplements to Freshly Drawn Blood on the Evolution of SPCA

Experiment	Without Thromboplastin			With Thromboplastin		
	Cl T	SPCA	Serum proth activity	Cl T	SPCA	Serum proth activity
	min	per cent	per cent	min	per cent	per cent
I*		121	4		300	0
II*	7½	46	13	<1	141	0
III†	8	44	36	<1	109	0
IV†	8½	74	32	<1	172	0

\* Serum withdrawn and oxalated 1 hour after coagulation.

† Serum withdrawn and oxalated immediately after coagulation.

*Effect of Retarding Coagulation.* Prothrombin activity and SPCA were measured in the serum from blood drawn and allowed to clot in siliconized apparatus according to the technic of Jacques et al.<sup>8</sup> Parallel with retardation of coagulation the serum showed abnormally high prothrombin activity and small amounts of SPCA.

The effect of heparin was also investigated. A fixed volume of venous blood was added to increasing concentrations of the anticoagulant (table 4). Although coagulation was retarded substantially in the first two samples, SPCA and prothrombin activity of their sera were unaffected. At larger concentrations of heparin SPCA was markedly reduced and residual prothrombin activity was abnormally high. It appears that the smaller concentrations of heparin increased antithrombin activity without affecting the speed or the amount of prothrombin conversion to thrombin. With larger amounts, this phase of coagulation was also disturbed.

To prove that the above observations were not attributable to heparin carried over into the serum, experiments were performed in which the anticoagulant was added to plasma mixtures in concentrations which would obtain if the serum contained all of the heparin unaltered. The anticoagulant failed to affect substantially the prothrombin activity of the plasma mixtures even in those concentrations

\* Thromboplastin added to oxalated dog serum always decreased its SPCA activity.

which retarded coagulation of fresh blood markedly \* It is accordingly evident that the above observations are not artifacts referable to the mere presence of heparin in the serum-plasma mixtures upon which prothrombin activities were determined

*Clot Accelerating Effect of Serum* 2.0 cc of blood from a normal subject were added to 0.1 cc of oxalated serum prepared in the usual manner but subjected to incubation (37 C) for two hours in order to assure maximal inactivation of thrombin Its SPCA activity was 173 The clotting time of the normal blood-serum mixture was 3½ minutes contrasted with a clotting time of the blood alone of 12 minutes This clot accelerating effect was not due to thrombin since the same serum added to oxalated normal plasma (1 part serum to 10 parts plasma) failed to induce clotting in 3 hours whereas approximately 5 units of thrombin to 1.0 cc of plasma clotted the mixture immediately It is noteworthy that the serum obtained from the whole

TABLE 4.—*SPCA Evolution and Residual Serum Prothrombin Activity in Heparinized Blood*

Hep added	Cl T	SPCA activity	Serum proth activity
units per 2 cc blood	min	per cent	
0	3	59	14
0.16	12	63	3.3
0.31	15	93	3.0
0.63	25	83	8.0
1.25	39	8	90.0
1.66	54	13	80

\* The plasma prothrombin activity of this blood was 90 per cent of normal.

blood-serum mixture whose clotting was accelerated did not show greater SPCA than the serum from the blood allowed to clot alone

*SPCA in Subjects Receiving Dicumarol* It was of interest to investigate the relation between the amount of prothrombin available for conversion to thrombin and the evolution of SPCA The concentration of this factor was followed in a subject who received dicumarol for treatment of myocardial infarction † The administration and withdrawal of the drug affected plasma prothrombin concentration and SPCA activity in the same direction (fig 3) Similar results were obtained in a normal dog which received dicumarol parenterally

Hypoprothrombinemic blood from 26 subjects with myocardial infarction who received dicumarol for treatment‡ was studied Their plasma prothrombin concentrations were between 3.8 and 10 per cent of normal (mean 7.1) The SPCA's ranged between 8.4 and 43 (mean 25, S D 9.7) The serum prothrombin activities were usually less than that of normal serum, never exceeding 4 per cent

\* It appears that the antithrombic action of heparin in these concentrations does not influence the prothrombin times markedly

† This patient showed no additional manifestation of phlebothrombosis or thromboembolism

‡ Part of a study supported by the U S P H on the effect of dicumarol on the thrombotic complications of myocardial infarction

In view of the observations that thromboplastin added to freshly drawn blood resulted in much greater SPCA, similar experiments were done on hypoprothrombinemic blood from 8 subjects. SPCA was increased substantially in only two instances, although clotting was accelerated not only in these cases but also in those where SPCA was unchanged.

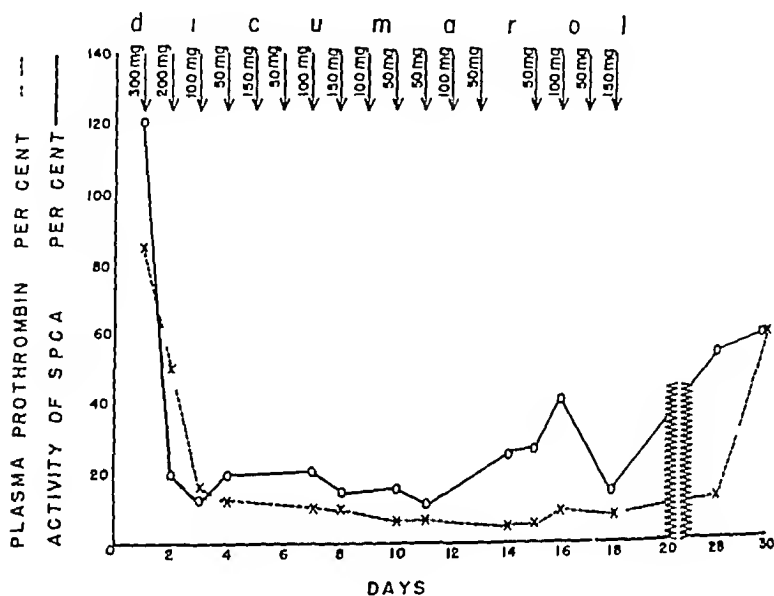


FIG 3

### DISCUSSION

The ranges of both serum prothrombin conversion accelerator and residual prothrombin activity in serum removed and oxalated one hour after coagulation have been delineated. The explanation for the wide variations is obscure. Two factors just be considered in SPCA evolution (1) speed of prothrombin conversion to thrombin and (2) the absolute amount of prothrombin converted. From the results obtained with mechanical agitation of, and with thromboplastin supplements to, clotting blood, it is evident that accelerating coagulation increases the amount of SPCA formed. Inhibition of clotting by large amounts of heparin or by siliconized apparatus suppresses SPCA evolution.

It should be pointed out that concomitant with accelerating coagulation more prothrombin is converted to thrombin as evidenced by less prothrombin activity remaining in the serum. Conversely more serum prothrombin is found when coagulation is retarded by silicone or large amounts of heparin. Whether under these conditions the substantial residual serum prothrombin is intimately related to the decreased SPCA or whether it, too, is simply a reflection of the retarded coagulation

requires elucidation. It would appear from experiments on dicumarolized blood that the total amount of prothrombin converted to thrombin plays an important role in the total amount of SPCA which can be evolved. This is predicated upon the assumption that dicumarol does not decrease, simultaneously with plasma prothrombin, a precursor of SPCA. The validity of this assumption, however, has yet to be substantiated.<sup>9</sup>

It is known that the coagulation of dicumarolized blood is prolonged under certain conditions.<sup>10, 11</sup> That this degree of retardation per se cannot, however, explain the low SPCA of serum from dicumarolized subjects is indicated by the inability, in most instances, of increasing SPCA development by accelerating coagulation of dicumarolized blood with thromboplastin.

It therefore appears that the amount of prothrombin converted to thrombin is one of the factors determining the amount of SPCA evolved. Another determinant is the velocity of prothrombin conversion. Both depend, *inter alia*, upon the concentrations of prothrombin and thromboplastin. That no correlation was evident between SPCA and the plasma-serum prothrombin activity difference in normal subjects may be referable to variation from individual to individual in the rate with which thromboplastin evolves after blood is shed.

An increment in SPCA, similar to that induced by thromboplastin added to freshly drawn normal blood, can also be produced by adding thromboplastin to serum which contains small amounts of prothrombin activity. That this enhancing effect is not obtainable if the serum is oxalated prior to the addition of the thromboplastin suggests that calcium is required for SPCA formation. It is striking that substantial increments in SPCA are thus obtained although only slight amounts of additional prothrombin are apparently consumed.

The *in vitro* action of heparin is of particular interest. Moderate amounts of the anticoagulant retard coagulation without, however, affecting either the amount of SPCA evolved or the amount of prothrombin which is consumed during coagulation. If anything, prothrombin consumption is increased, probably as a result of the greater interval provided by the retarded coagulation for the reaction to proceed. Although the anticoagulant is said to have antiprothrombic<sup>12</sup> as well as antithrombic<sup>13</sup> properties, moderate doses seem to act by enhancing the latter. Larger doses retard coagulation also by inhibiting the evolution of thromboplastin from platelets or by otherwise preventing the conversion of prothrombin to thrombin. Concomitant with this, SPCA evolution falls off.

Of fundamental importance is the ability of serum to accelerate the coagulation of whole blood. Its practical significance derives from the realization that this may be the mechanism underlying clot propagation *in vivo*.

Thrombin has been excluded as the clot promoting agent in serum. Since, however, it has been shown that thromboplastin is not consumed in the process of blood coagulation, it is possible that the clot accelerating action of serum is due to unconsumed thromboplastin liberated during blood coagulation. That thromboplastin can thus be implicated is highly unlikely since serum has only slight effect on the clotting time of hemophilic blood, which is very sensitive to thromboplastin.<sup>14</sup>

The value of dicumarol in the prevention and treatment of thromboembolism may well be related to its interference with SPCA evolution

### SUMMARY

- 1 The evolution of a factor in serum which accelerates prothrombin conversion to thrombin has been studied in normal subjects
- 2 Mechanical agitation of fresh blood or the addition of thromboplastin supplements increases the amount of SPCA evolved and decreases the amount of prothrombin activity remaining in the serum
- 3 Retarding coagulation by large doses of heparin or by handling the blood with siliconized apparatus decreases SPCA evolution and increases residual serum prothrombin activity
- 4 Hypoprothrombinemic blood resulting from dicumarol administration evolves subnormal amounts of SPCA during its coagulation

### REFERENCES

- <sup>1</sup> DE VRIES, A. ALEXANDER B., AND GOLDSTEIN, R. A factor in serum which accelerates the conversion of prothrombin to thrombin I. Its determination and physiologic and biochemical properties. *Blood* 4, 247, 1949
- <sup>2</sup> WARE, A. G. AND SREOGERS, W. H. Serum accelerator globulin. Quantitative determination, purification and properties. *Fed Proc* 7, 131, 1948
- <sup>3</sup> OWREN, P. A. Coagulation of blood. Investigations on a new clotting factor. *Acta Med. Scand. Suppl* 194, 1, 1947
- <sup>4</sup> ALEXANDER B., AND DE VRIES, A. A factor in serum which accelerates the conversion of prothrombin to thrombin III. Its relationship to the coagulation defect of thrombocytopenic blood. *Blood* this issue, page 747
- , AND —. Studies on hemophilia V. The coagulation defect in hemophilia with special reference to inadequate prothrombin consumption. *Blood* this issue, page 752.
- <sup>6</sup> POHLER, F. J. AND TAYLOR, F. H. L. The coagulation defect in hemophilia. The effect of intramuscular administration of a globulin substance derived from normal human plasma. *J Clin Investigation* 16, 741, 1937
- <sup>7</sup> CHARGAFF, E. J. Studies on the mechanism of the thromboplastic effect. *J Biol Chem.* 173, 253, 1948.
- <sup>8</sup> JACQUES, L. B. FIDLAR, E., FELDSTED, E. T. AND McDONALD, A. G. Silicones and blood coagulation. *Canad M. A. J.* 55, 26, 1946
- <sup>9</sup> OWEN, C. A. AND BOLLMAN, J. L. Prothrombin conversion factor in dicumarol plasma. *Proc. Soc. Exper Biol & Med* 67, 231, 1948
- <sup>10</sup> WASSERMAN, L. R., AND STATT, D. Clinical observations on effect of 3,3'-methylenebis (4-hydroxycoumarin). *Am J M. Sc.* 206, 466, 1943
- <sup>11</sup> DAVIDSON, C. S. AND McDONALD, H. A critical study of the action of 3,3'-methylenebis (4-hydroxycoumarin) (Dicoumarin). *Am J M. Sc.* 205, 24, 1943
- <sup>12</sup> BRINKHOUT, K. M., SMITH, H. P., WARNER, E. D. AND SREOGERS, W. H. Inhibition of blood clotting. An unidentified substance which acts in conjunction with heparin to prevent the conversion of prothrombin into thrombin. *Am J Physiol* 125, 683, 1939
- <sup>13</sup> HOWELL, W. H., AND HOLT, E. Two new factors in blood coagulation—heparin and pro-antithrombin. *Am J Physiol* 47, 328, 1918
- <sup>14</sup> QUICK, A. J. Studies on the enigma of the hemostatic dysfunction of hemophilia. *Am. J. M. Sc.* 224, 272, 1947

# A FACTOR IN SERUM WHICH ACCELERATES THE CONVERSION OF PROTHROMBIN TO THROMBIN

## III ITS RELATIONSHIP TO THE COAGULATION DEFECT OF THROMBOCYTOPENIC BLOOD

By BENJAMIN ALEXANDER, M D , AND ANDRE DE VRIES, M D \*

*With the technical assistance of EUNICE ADDELSON*

THE EXACT role of the platelet in blood coagulation is the subject of considerable controversy. Although thrombocytopenic plasma exhibits retarded coagulation, a prolonged clotting time is rare in thrombocytopenic purpura.<sup>1-3</sup> This has been explained by the theory that even in severe thrombocytopenia sufficient thromboplastin is elaborated to produce normal coagulation.<sup>4</sup> In any event, the hemorrhagic manifestations of thrombocytopenic purpura have generally been ascribed either to a great reduction in blood platelets, to capillary dysfunction<sup>5</sup> or to inadequate clot retraction rather than to abnormalities in coagulation itself. It is the purpose of this paper to present observations which indicate that the coagulation of thrombocytopenic blood is profoundly disturbed.

In a previous communication<sup>6,7</sup> an agent was described in serum which accelerates the conversion of prothrombin to thrombin in the presence of thromboplastin plus calcium. While insufficient data are available to establish the identity or non-identity of this substance with other factors reported to have similar attributes,<sup>8,9</sup> some of its biochemical and physiologic properties have been described, a method for its determination given, and its elaboration in the coagulation of normal blood delineated.

### METHODS

The agent, serum prothrombin conversion accelerator (SPCA), is measured by the enhancement in per cent of the prothrombin activity of normal oxalated plasma induced by the admixture to it of serum obtained from the blood in question one hour after coagulation.<sup>6</sup> Before the test, the serum is oxalated and incubated for one half hour in order to inactivate thrombin.

The prothrombin activities of plasma and serum were determined by modifications of the one stage procedure<sup>6</sup>; coagulation time was measured by a modification of the Lee and White technic.<sup>10</sup> Platelets were enumerated by the method of Rees and Ecker,<sup>11</sup> and bleeding time was determined by the Duke method.<sup>12</sup>

### RESULTS

Ten subjects† with thrombocytopenic purpura were studied (table 1). All had platelet counts below 100,000 per mm.<sup>3</sup> The mean SPCA activity was 33 per cent in contrast to 99 for 95 normal subjects previously reported (7). The residual serum

From the Medical Research Laboratory, Beth Israel Hospital and the Department of Medicine, Harvard Medical School, Boston, Mass.

This study supported by a grant from the Commonwealth Fund.

\* Aided by a fellowship from the Rothschild Hadassah Hospital, Jerusalem.

† We are indebted to Drs. William B. Castle and Robert Epstein for generously making available some patients and facilities of the Thorndike Memorial Laboratory, Boston City Hospital, Boston.

prothrombin activity averaged 50 per cent\* compared with 6 per cent for normal individuals. The algebraic differences between plasma and serum prothrombin activities averaged 37.5, whereas in normals they average 90+.

TABLE 1.—*Prothrombin Consumption and Evolution of Serum Prothrombin Conversion Accelerator in Coagulation of Thrombocytopenic Blood*

Subj	Disease	Plat no	Bleed time	Clot. time	Prothrom activity per cent			SPCA
					Plasm	Ser	Plas Ser	
		thous per mm <sup>3</sup>	min	min				per cent
R. P o+	Idiopathic thromb purpura	13	4	13	97	47	50	19
H. J o+	Idiopathic thromb purpura	22	>10	12	120	77	43	22
D o→	Idiopathic thromb purpura	11	14	11½	100	55	45	37
M. o+	Idiopathic thromb purpura	97	—	11	71	10	61	53
C. G o+	Idiopathic thromb purpura	70	4½	9	141	127	14	18
M. S o+	Idiopathic thromb purpura	30	30	10	120	80	40	24
B. M. o+	Cirrhosis, splenomegaly	69	5½	14	96	25	71	34
S. S o→	Hodgkin's dis Nitrogen mustard therapy	76	6	11	60	67	0	22
W. o+	Gaucher's disease	67	—	16	65	58	7	25
X. o+	Multiple myeloma	94	—	—	58	47	11	58

\* All values corrected for dilution with oxalate

TABLE 2.—*Effect of Normal Plasma, Platelets or Thromboplastin on Coagulation of Thrombocytopenic Blood*  
Patient M. S. Idiopathic Thrombocytopenic Purpura

	Platelet	Clotting time	SPCA	Serum proth activity	Plasma minus serum proth activity
	thousands per mm <sup>3</sup>	min	per cent	per cent	per cent
Blood alone	60	14½	28	67	15
2 cc. blood plus 0.1 cc. normal plasma	74	5½	34	52	30
2 cc. blood plus 0.2 cc. normal plasma	87	5½	47	44	38
2 cc. blood plus platelets from 0.2 cc. normal plasma*		4	56	36	46

Patient C. G. Idiopathic Thrombocytopenic Purpura

Blood alone	70	9	18	115	3
2 cc. blood plus 0.2 cc. thromboplastin sol †	70	<1	165	17	101

\* 1 cc. oxalated plasma was centrifuged at 3000 r.p.m. for 30 minutes. The supernatant plasma was decanted and the sediment suspended by stirring and vigorous shaking in 1 cc. of physiologic saline. 0.1 cc. of the mixture was added to 2 cc. of the patient's blood.

† Thromboplastin solution prepared from Difco commercial thromboplastin as for prothrombin determination.\*

No strict correlation was evident between the bleeding time or platelet count on the one hand and the SPCA or residual serum prothrombin activity on the other,

\* Normal plasma is considered to have 100 per cent prothrombin activity

although those subjects with the highest platelet counts seemed to have the highest SPCA activities. The coagulation times of most of the patients were within the accepted range of normality.

The addition of normal oxalated plasma, platelets or thromboplastin extract to shed thrombocytopenic blood accelerated coagulation, increased prothrombin consumption, and increased the amount of SPCA evolved (table 2).

Of considerable interest are the observations in one subject with idiopathic thrombocytopenic purpura before and after splenectomy (table 3). Despite the fact that the platelet count and the bleeding time returned to normal following the

TABLE 3—Platelet Count, Serum Prothrombin, and SPCA Following Splenectomy for Idiopathic Thrombocytopenic Purpura

Subject M. S.

Date	Platelets	Cl T	Bl T	Prothrombin		SPCA
				Plasma	Serum	
	thousands per mm <sup>3</sup>	min	min	per cent	per cent	per cent
6/11/48	30	10	32+	100	73	24
6/22	60	14½	—	82	37	39
6/30	30	—	—	—	—	—
7/1	Splenectomy					
7/1*	59	—	25	—	43	22
7/2	188	11	3½	71	39	8
7/3	178	—	3½	81	16	29
7/6	183	—	4	126	15	43
7/8	320	—	2	92	19	30
7/10	226	18	3	94	31	52
7/16	312	—	31	—	—	—
8/5	200	—	12	—	—	—
8/18	60	—	9	—	—	—
8/24	48	—	—	58	46	55

Splenic artery blood obtained during the operation

procedure, there was practically no change in the SPCA. Residual serum prothrombin did, however, decrease somewhat, but as the patient relapsed about one month after operation, it again increased.

#### DISCUSSION

Evolution of SPCA during coagulation is enhanced by supplements of thromboplastin to, or by mechanical agitation of, clotting blood.<sup>7</sup> Conversely, it is markedly reduced by inhibiting coagulation by exposing blood to siliconized surfaces, a condition which interferes with thromboplastin elaboration. Concomitantly, residual serum prothrombin activity is greatly increased.

The similar observations in thrombocytopenia indicate that a decreased number of platelets is associated with insufficient evolution of thromboplastin. This results in abnormally small prothrombin conversion to thrombin associated with inadequate SPCA evolution, as a consequence of which the conversion of additional prothrombin to thrombin is retarded. That the clotting times were essentially



normal despite the clotting defect reflects the lack of sensitivity of this test. Similarly in dicumarolized plasma the coagulation time is, more often than not, normal while SPCA is small.<sup>7</sup> And in hemophilia<sup>12</sup> comparable abnormalities in residual prothrombin activity and SPCA elaboration are observed even when the clotting time is restored toward normal by the addition of normal plasma or thromboplastin extracts. These observations are understandable when it is realized that in the coagulation of blood only a very small fraction of the total plasma prothrombin need be converted to thrombin to give a normal clotting time.<sup>14</sup>

From the experiments on one subject before and after splenectomy it appears that restoration of the platelet count to normal did little to remedy the clotting defect. What relation this had to the prompt relapse of the thrombocytopenia with clinical manifestations of bleeding is obscure and demands further exploration. It seems that although the patient had a normal number of circulating platelets following operation, they may not have been qualitatively satisfactory for rectifying the defect in coagulation. This is substantiated by the fact that the addition of normal plasma or platelets therefrom to the blood of this same subject corrected the abnormality. Such a concept is in accord with interpretations by Aggeler et al. of evidence regarding variability in the functional capacity of platelets.<sup>15</sup>

The significance of these abnormalities in the pathogenesis of the hemorrhagic phenomena of thrombocytopenic purpura requires further investigation. According to Allen et al.<sup>16</sup> a circulating heparin-like anticoagulant may be present in idiopathic thrombocytopenic purpura. The clotting defect observed by us in this disease cannot be attributed to heparin since we found<sup>7</sup> that the addition of moderate amounts of heparin to freshly drawn normal blood so as to retard coagulation substantially failed to inhibit SPCA evolution or prothrombin conversion to thrombin.

### SUMMARY

The sera from thrombocytopenic blood show abnormally large residual prothrombin activity and small amounts of prothrombin conversion accelerator. The addition of normal platelets or thromboplastin corrects these abnormalities. In one subject the clotting defect persisted despite temporary remission of the thrombocytopenia consequent to splenectomy.

### REFERENCES

- <sup>1</sup> HESS, A. F. A consideration of the reduction of blood platelets in purpura. *Proc. Soc. Exper. Biol. & Med.* 14, 96, 1917.
- <sup>2</sup> LE Sourd, L. AND PAONIEZ, P. Recherches sur le rôle des plaquettes sanguine ou hematoblastes dans la coagulation du sang. *J. de physiol. et de path. gen.* 11, 1, 1909.
- <sup>3</sup> NYGAARD, K. K., GIFFIN, H. Z. AND PEMBERTON, J. Coagulability of the blood in essential thrombocytopenia and other diseases associated with thrombocytopenia, with observations following splenectomy. *Proc. Staff Meet. Mayo Clin.* 15, 753, 1940.
- <sup>4</sup> QUICK, A. J. *The Hemorrhagic Diseases*. Springfield, Ill.: Charles C. Thomas, 1942, p. 85.
- <sup>5</sup> MACFARLANE, R. G. Critical review. The mechanism of hemostasis. *Quart. J. Med.* 11, 1, 1941.
- <sup>6</sup> DE VRIES, A., ALEXANDER, B. AND GOLDSTEIN, R. A factor in serum which accelerates the conversion of prothrombin to thrombin. I. Its determination and some physiological and biochemical properties. *Blood* 4, 247, 1949.

- <sup>7</sup> ALEXANDER, B., DE VRIES, A., AND GOLDSTEIN, R. A factor in serum which accelerates the conversion of prothrombin to thrombin II. Its evolution with special reference to the influence of conditions which affect blood coagulation. *Blood*, this issue, page 739.
- <sup>8</sup> OWREN, P. A. Coagulation of blood. Investigations on a new clotting factor. *Acta Med Scand Suppl* 194, 1, 1947.
- <sup>9</sup> WARR, A. G., AND SEEGER, W. H. Serum accelerator globulin. Quantitative determination, purification and properties. *Fed. Proc.* 7, 131, 1948.
- <sup>10</sup> FOHLE, F. J., AND TAYLOR, F. H. L. The coagulation defect in hemophilia. The effect in hemophilia of intramuscular administration of a globulin substance derived from normal human plasma. *J. Clin. Investigation* 16, 741, 1937.
- <sup>11</sup> REES, H. M., AND ECKER, E. E. An improved method for counting blood platelets. *J. A. M. A.* 80, 621, 1923.
- <sup>12</sup> DUKE, W. W. The relation of blood platelets to hemorrhagic disease. Description of a method for determining the bleeding time and coagulation time and report of three cases of hemorrhagic disease relieved by transfusion. *J. A. M. A.* 55, 1185, 1910.
- <sup>13</sup> ALEXANDER, B., AND DE VRIES, A. Studies on hemophilia. V. The coagulation defect in hemophilia with particular reference to the conversion of prothrombin to thrombin and the evolution of the prothrombin conversion accelerator. *Blood*, this issue, page 752.
- <sup>14</sup> SEEGER, W. H., AND SMITH, H. F. Factors which influence the activity of purified prothrombin. *Am. J. Phys.* 137, 348, 1942.
- <sup>15</sup> AGGELER, P. M., HOWARD, J., AND LUCIA, S. P. Platelet counts and platelet function. *Blood* 1, 472, 1946.
- <sup>16</sup> ALLEN, J. G., BOGARDES, G., JACOBSON, L. O., AND SPURR, C. L. Some observations on bleeding tendency in thrombocytopenic purpura. *Ann. Int. Med.* 27, 382, 1947.

## STUDIES ON HEMOPHILIA

### V THE COAGULATION DEFECT IN HEMOPHILIA WITH PARTICULAR REFERENCE TO THE CONVERSION OF PROTHROMBIN TO THROMBIN AND THE EVOLUTION OF THE PROTHROMBIN CONVERSION ACCELERATOR

BENJAMIN ALEXANDER, M D , AND ANDRE DE VRIES, M D \*

*With the technical assistance of EUNICE ADDELSON*

MOST investigators agree that the conversion of prothrombin to thrombin is retarded in the coagulation of hemophilic blood. This is reflected in the high prothrombin activity of hemophilic serum.<sup>1, 2</sup> The clotting of hemophilic blood can be accelerated by the addition of thromboplastin, normal plasma, or fractions thereof. Brinkhous<sup>1</sup> and Quick<sup>2</sup> reported that such additions simultaneously increase prothrombin consumption. The latter author used this effect as a basis for assay of the antihemophilic activity of normal plasma.

Recently, substances have been described which, arising in blood during its coagulation, accelerate the conversion of prothrombin to thrombin in the presence of thromboplastin plus calcium.<sup>3, 4</sup> Their evolution and physiologic properties help explain the autocatalytic process underlying thrombin formation. In a previous publication<sup>5</sup> and elsewhere in this issue<sup>6, 7</sup> we have reported on serum prothrombin conversion accelerator (SPCA), delineating its evolution under various conditions in normal subjects and in patients with thrombocytopenic purpura. This report, concerning similar studies in hemophilia, presents data indicating that in the elaboration of this clotting factor, also, the coagulation of hemophilic blood is abnormal.

#### METHODS

Plasma and serum prothrombin activities and SPCA were determined by methods previously described.<sup>8</sup> In normal subjects<sup>8</sup> serum prothrombin activity ranges between 0-32 (mean 6.4), SPCA ranges between 43-271 (mean 99). In some experiments prothrombin was simultaneously measured by the modified two stage method of Jaques<sup>9</sup> using Parke Davis Topical Thrombin as our standard.

Clotting time was determined by a modification of the Lee and White method.<sup>10</sup> In our experience the value in 90 normal individuals was between 4 and 12 minutes (mean 7.7 S.D., 1.72).

#### RESULTS

*Serum Prothrombin Activity and SPCA in Hemophilia* The prothrombin activities of sera removed and oxalated one hour after coagulation from hemophilic blood are abnormally high. This is in accord with the observation of others.<sup>1</sup> Conversely, the SPCA activities are abnormally low (table 1). No correlation was evident between the coagulation time and these serum entities.

From the Medical Research Laboratory, Beth Israel Hospital and the Department of Medicine, Harvard Medical School, Boston, Mass.

This study supported by a grant from the Commonwealth Fund.

\* Aided by a Fellowship from the Rothschild Hadassah University Hospital, Jerusalem.

TABLE 1.—*SPCA and Serum Prothrombin Activity in Hemophiliacs*

Subj †	Cl T	One stage proth activity, per cent*		SPCA
		Plas	Ser	
R. R.	min			
I. G.	110			per cent
J. G.	90	60	77	16
W. H. †	164	96	118	0
N. B.	43	—	90	0
R. W.	200	52	53	8
P. W.	87	66	63	21
A. Z.	62	80	73	22
R. S.	43	53	54	35
	45	72	53	16
Average		92	75	19
		71	73	15

\* Compared with prothrombin activity of a pool of normal plasma (10 normal subjects) which is considered to be 100 per cent.

† This patient received a blood transfusion one day before the test. All other subjects received no therapy for at least three days before the test.

‡ We are grateful to Drs. William B. Castle and Robert Epstein of the Thorndike Memorial Laboratory, Boston, for making some of their hemophiliacs available for study.

TABLE 2.—*Effect of Thromboplastin Supplements on Clotting Time, Serum Prothrombin Activity, and SPCA in Hemophiliacs*

Added thromboplastin*	Cl T	Ser proth activity	SPCA
Subject R. R. Experiment 1			
cc	min	per cent	per cent
0	150	126	11
0.0005	3	132	0
0.20	<20 sec.	<5	144
Experiment 2			
0	90	93	0
0.00001	44	76	0
0.0005	6.5	77	0
0.001	5	72	0
Subject I. G.			
0	32	93	11
0.1	<4	58	49
Subject R. S.			
0	138	130	0
0.1	30 sec.	21	149

\* Disco thromboplastin prepared as for prothrombin determination.\* The figures indicate the amount of this thromboplastin extract contained in 0.2 cc. of saline solution, added to 2.0 cc hemophiliac blood.

*Effect of Accelerating Coagulation on Residual Serum Prothrombin Activity and SPCA Evolution* In normal subjects, accelerating coagulation by the addition of thromboplastin increases SPCA and removes the last traces of serum prothrombin activity. Restoring the clotting time of hemophilic blood to normal *in vitro* by the addition

TABLE 3 — *Effect of Accelerating Coagulation of Hemophilic Blood by Additions of Normal Plasma in Vitro and in Vivo on Residual Serum Prothrombin Activity and on SPCA Evolution*

Subj	Norm plas added to 2.0 cc. hemoph blood	Cl T	One stage ser proth activity	SPCA
In Vitro				
	cc.†	min	per cent	per cent
R. R.	0	98	74	7
	0 0005	38	78	0
	0 001	23	62	0
	0 010	10	60	0
	0 10	8	43	11
I. G.	0	27	95	12
	0 10	14	70	15
	0 20	9	49	42
J. G.	0	85	130	—
	0 001	53	124	—
	0 005	33	124	—
	0 010	17	128	—
	0 10	7	115	—
In Vivo				
	Plasma intraven.*			
	cc			
R. R.	0	60	94	13
	180	15	42	18
	0	120†	68	8
	700	11	25	32
I. G.	0	53	108	6
	150	14	58	30

\* Citrated plasma. In the *in vivo* experiments determinations were done on blood drawn 10 minutes after infusion was completed.

† The amounts of normal plasma added were contained in a volume of 0.1 cc. of a saline solution

of small amounts of thromboplastin fails to lower residual serum prothrombin activity appreciably, SPCA concentration is also unaffected. When, however, larger amounts of thromboplastin are supplied, both prothrombin consumption and SPCA evolution may be greatly increased, attaining normal values (table 2). It is striking, however, that in two subjects substantial amounts of serum prothrombin activity were still demonstrable although the parent blood had clotted in 180

seconds or less This is in marked contrast to what was observed in normal subjects \*

Restoration of the clotting time of hemophilic blood toward normal by the *in vitro* or *in vivo* addition of normal plasma decreases residual serum prothrombin activity substantially in some cases but it rarely reaches normal values (table 3)

TABLE 4.—*Residual Serum Prothrombin Activity in Hemophilia as Determined by the One Stage and Two Stage Procedures Subject R. R.*

Prothrombin						
	Cl T	One stage		Two stage		SPCA
		Plasma	Serum	Plasma	Serum*	
	min	per cent	per cent	units	units	per cent
Blood spont. clotted, no additions	60	50	55	121	<55†	17
2.0 cc. blood plus 0.0001 cc. thromboplastin in 0.1 cc. saline	18		70	121	<55†	0
Blood spont. clotted no additions	114	50	65	114	24†	13
2 cc. blood plus 0.01 cc. normal plasma in 0.1 cc. saline	13½		50	114	37†	50

\* 1 hour after coagulation

† How much less could not be ascertained because less dilution would have been required which would introduce errors due to antithrombin activity

‡ Computed by subtracting plasma prothrombin from the prothrombin determined in a one to one mixture of plasma plus serum This was an attempt to circumvent the above difficulty

TABLE 5.—*Accelerating Effect of Normal Serum and Plasma on Coagulation of Hemophilic Blood Hemophilic Subject R. R.*

Oxalated plasma and oxalated serum from normal subject. The SPCA activity of the serum was 101.

Added to 2.0 cc. hemoph blood	Cl T
<i>cc</i>	<i>min</i>
—	
0	60
0.001 serum	49
0.01 serum	33
0.001 plasma	33
0.01 plasma	21

SPCA, however, rises only slightly, even when as much as 700 cc of normal plasma are infused It is noteworthy that in other individuals coagulation may thus be accelerated without any demonstrable change in residual serum prothrombin activity (J. G., table 3) Also, even when serum prothrombin activity is decreased by the addition of normal plasma (R. R., table 3), the change is far less marked than the decrease in the clotting time

*Effect of Normal Serum on Coagulation of Hemophilic Blood* The addition of normal serum, containing substantial SPCA activity, to hemophilic blood (table 5)

accelerated coagulation only slightly as compared with the clot promoting effect of the parent plasma

*Residual Serum Prothrombin Activity in Hemophilia as Determined by the One and the Two Stage Technics* Hemophilic plasma, and sera obtained one hour after coagulation were subjected to simultaneous prothrombin determination by both the one stage and the two stage technics. Whereas by the one stage procedure serum prothrombin activity was no less (and occasionally was even more) than that of its parent plasma, by the two stage method serum prothrombin activity was markedly less (table 4)

#### DISCUSSION

That hemophilic sera contain large amounts of prothrombin has been repeatedly observed.<sup>1, 2</sup> The abnormally small evolution of serum prothrombin conversion accelerator during the coagulation of hemophilic blood indicates an additional coagulation defect which may play a significant role in the pathogenesis of the hemorrhagic phenomena in this disease. It is striking that in thrombocytopenic purpura, also, both high residual serum prothrombin activity and low SPCA are found.<sup>7</sup> Similarly, SPCA concentrations are decreased in the sera of blood rendered hypoprothrombinemic by the administration of dicumarol.<sup>6</sup> The possibility must be considered that subnormal SPCA elaboration is the common denominator underlying the hemorrhagic tendency of these various disorders.

The addition of small amounts of normal plasma can accelerate the coagulation of hemophilic blood without appreciably affecting the apparent prothrombin activity remaining in the serum. This clot promoting effect is not due to thrombin evolved from the small amount of prothrombin contained in the added normal plasma since *prothrombin free* plasma also shows full clot promoting activity.<sup>10</sup> It appears that the normal plasma acts by accelerating the evolution of thromboplastin.<sup>2</sup> This induces prothrombin conversion to thrombin in amounts sufficient to clot the blood in a relatively normal time. Since no more than 2 of the approximately 250 units of thrombin which can be formed in 1 cc of normal plasma are required for this purpose,<sup>11</sup> it is understandable how the coagulation time of hemophilic blood can be brought within normal limits by small additions of normal plasma, or indeed of thromboplastin, without significantly decreasing residual serum prothrombin. This point has been emphasized by Quick.<sup>2</sup>

The above facts may also explain the frequent clinical observation that hemophiliacs may continue to bleed despite relatively normal coagulation times induced by therapy with blood, plasma, or plasma fractions.

Quick has reported a method of assaying antihemophilic activity of plasma by its ability to induce substantial prothrombin consumption when added to hemophilic blood.<sup>2</sup> This method is less sensitive than the procedure of Alexander and Landwehr<sup>12</sup> which is based upon reduction in the coagulation time since clotting can be significantly accelerated by much smaller quantities of normal plasma than are required for significant alterations in prothrombin consumption.

An additional criticism of Quick's method arises from the discrepancy in serum prothrombin activity as determined by the one stage and two stage procedures.

This is of great interest and demands further investigation. Until it is satisfactorily explained, computations of prothrombin consumption from differences between plasma and serum prothrombin activity are to be interpreted with caution.

The addition of large amounts of thromboplastin to normal blood results in serum which is devoid of prothrombin activity.<sup>6</sup> That, in contrast, the sera of some hemophiliacs retain considerable prothrombin activity despite the fact that coagulation is extremely rapid consequent to the addition of thromboplastin indicates that clotting is still abnormal and suggests that deficient elaboration of thromboplastin is not the sole defect, at least in some cases, in the coagulation of hemophilic blood. The recent evidence regarding anticephalin<sup>13</sup> and an anticoagulant in the nature of an immune body<sup>14</sup> may bear on this point.\*

### CONCLUSIONS

It has been confirmed that hemophilic serum exhibits considerable prothrombin activity. The clotting time may be restored to normal by the addition of normal plasma or thromboplastin without affecting residual serum prothrombin activity significantly.

2. In contrast to normal, hemophilic serum is relatively incapable of accelerating the conversion of plasma prothrombin to thrombin in the presence of thromboplastin and calcium. This defect also persists despite acceleration of coagulation to normal by additions of normal plasma or thromboplastin.

3. Serum from hemophilic blood which clots rapidly in the presence of large supplements of thromboplastin may still retain substantial prothrombin activity, whereas under the same conditions normal serum is devoid of prothrombin activity.

4. There is a marked discrepancy between the prothrombin activity of hemophilic serum as determined by the one and the two stage procedures.

### REFERENCES

- <sup>1</sup>BRINKHOUTS, K. M. A study of the clotting defect in hemophilia. The delayed formation of thrombin. *Am J M. Sc.* 198: 509, 1939.
- <sup>2</sup>QUICK, A. J. Studies on the enigma of the hemostatic dysfunction of hemophilia. *Am J M. Sc.* 214: 272, 1947.
- <sup>3</sup>WARE, A. G., MURPHY, R. C., AND SEEGER, W. H. The function of Ac globulin in blood clotting. *Science* 106: 618, 1947.
- <sup>4</sup>OWREN, P. A. Coagulation of blood. Investigations on a new clotting factor. *Acta Med. Scand.* Suppl. 194: 1, 1947.
- <sup>5</sup>DE VRIES, A., ALEXANDER, B. AND GOLDSTEIN, R. Studies on a factor in serum which accelerates the conversion of prothrombin to thrombin. I. Its determination and some physiological and biochemical properties. *Blood* 4: 247, 1949.
- <sup>6</sup>ALEXANDER, B., DE VRIES, A. AND GOLDSTEIN, R. Studies on a factor in serum which accelerates the conversion of prothrombin to thrombin. II. Its evolution with special reference to the influence of conditions which affect blood coagulation. *Blood* this issue, page 739.
- <sup>7</sup>—, AND —. Studies on a factor in serum which accelerates the conversion of prothrombin to thrombin. III. Its relation to the coagulation defect in thrombocytopenic blood. *Blood* this issue, page 747.
- <sup>8</sup>JACQUES, L. B. A quantitative method of assay for thrombin and prothrombin. *J. Physiol.* 100: 275, 1941.

\* In this connection it should be noted that the subjects of table 2 had been receiving repeated infusions of normal plasma over a considerable period.<sup>15</sup>



- <sup>9</sup> POHLE, F J , AND TAYLOR, F H L. The coagulation defect in hemophilia. The effect in hemophilia of intramuscular administration of a globulin substance derived from normal human plasma. *J Clin Investigation* 16 741, 1937
- <sup>10</sup> ALEXANDER, B AND DE VRIES A. Human prothrombin. Quantitative studies on the plasma labile factor and the restorative effect of normal, hypofibrinogenemic and hemophilic plasma on the prothrombin of stored plasma. *J Clin Investigation* 28 24 1949
- <sup>11</sup> SEEGERS, W H AND SMITH, H F. Factors which influence the activity of purified thrombin. *Am. J Physiol* 137 348, 1942.
- <sup>12</sup> ALEXANDER, B , AND LANDWEHR, G. Studies on hemophilia. II. The assay of the antihemophilic clot promoting principle in normal human plasma with some observations on the relative potency of certain plasma fractions. *J Clin Investigation* 27 98, 1948
- <sup>13</sup> TOCANTINS, L. M. Demonstration of antithromboplastic activity in normal and hemophilic plasma. *Am J Physiol* 139 265, 1943
- <sup>14</sup> CRADDOCK, C. G , JR , AND LAWRENCE J S. Hemophilia. A report of the mechanism of the development and action of an anticoagulant in two cases. *Blood* 2. 505, 1947
- <sup>15</sup> ALEXANDER, B AND LANDWEHR, G. Studies on hemophilia. I. The control of hemophilia by repeated infusion of normal human plasma. *J. A. M. A.* 138 174, 1948



for persistent diarrhea but steadily became more feeble. The treatment was chiefly dietetic, and radiation treatment was not given. The leukocyte count remained low in October 1945 for instance 2,160 per cu mm, with lymphocytes 74 per cent, in August 1947 4,500 with lymphocytes 24 per cent. The sternal marrow was heavily infiltrated with lymphocytes. In September 1947 he died. Only partial necropsy was performed. A firm annular infiltration extending a little above and below the pylorus was found in the stomach. Its surface was somewhat ulcerated. There were numerous lymph nodes in the mesentery. The liver was not enlarged and the spleen weighed 315 Gm. Histologic examination revealed an adenocarcinoma in the pyloric region. There were histologic changes in the spleen, liver, mesenteric lymph nodes and colon consistent with lymphatic leukemia. In the liver only very slight periportal infiltrations were noted. There were no metastases in the organs examined.

*Comment* In this case a typical lymphatic leukemia with characteristic blood picture was demonstrated in 1941. The anamnestic data make it probable that the condition had already existed for a number of years. From 1941 until the patient's death in 1947 the leukemia had, in spite of very moderate radiation treatment, remained aleukemic. In the last two years, there had been gastric and intestinal symptoms and increasing cachexia, and at the necropsy an ulcerated adenocarcinoma was found in the region of the pylorus, but lymphatic infiltrations in the lymph nodes, liver, spleen and colon were present. No other organs were examined.

Though the rather protracted aleukemic phase after a definite leukemic beginning is unusual, there can hardly be any doubt that the patient had a genuine leukemia. The long duration of the leukemic symptoms makes it overwhelmingly probable that the cancer of the stomach was a disease of later origin.

Besides this case, we have had occasion to observe 2 cases of cancer of the stomach, in which the first diagnosis both clinically and hematologically was chronic lymphatic leukemia, but in which the leukemic symptoms disappeared little by little as the cancer of the stomach developed. The first of these has already been published,<sup>4</sup> and shall therefore only be briefly recapitulated here.

*P. I. P.* (Radium Center 2114/35) farmer born 1879. In 1935, there appeared a plum-sized swelling of lymph nodes in his axillae and groins, and at the same time he began to suffer from increasing fatigue. The lymph node swelling was constant for four months and he was admitted to the Radium Center. There was no enlargement of the liver or spleen but roentgen examination of the lungs showed enlarged hilar glands. (For the blood findings, see the condensed table 1.) Most of the lymphocytes were small, rich in chromatin, but with very sparse cytoplasm. A few were atypical with lobulated or bi-nucleated nuclei. There were a number of disintegrated cells. A brief series of spray x radiations were given seemingly with good effect. The man remained perfectly well until the fall of 1937 when dyspeptic symptoms began to develop. In another hospital exploratory laparotomy was performed and a large inoperable carcinoma of the stomach invading the liver was found. In January 1938, he was readmitted to the Radium Center to be treated with roentgen ray for the tumor of the stomach. It was treated locally. At that time there was no swelling of the peripheral lymph nodes and no enlargement of the spleen. He died November 1938. Necropsy showed a large carcinoma of the stomach, with invasion of the left lobe of the liver and metastases to small glands along the lesser curvature but otherwise no metastases were found. There were no other glandular swellings and the spleen was not enlarged. Histologically the tumor of the stomach was a typical adenocarcinoma. There were no signs of lymphatic leukemia in the liver, spleen or kidneys. In the bone marrow there were a few very small groups of small lymphocytes but otherwise normal erythromyelopoiesis.

*Comment* The results of some of the blood examinations are shown in Table 1, further details may be found elsewhere.<sup>4</sup> It will be seen that when the patient was admitted in 1935, the blood picture showed a marked lymphatic reaction and the

lymphocytes showed morphologic abnormalities such as are often seen in lymphatic leukemia. In the course of a few months, the total leukocyte count dropped to normal values and at the same time the proportion of lymphocytes decreased, until at the time of the patient's death, there was a marked relative and absolute lymphopenia. In this case, it is difficult to say when the tumor in the stomach began to develop. When laparotomy was performed in 1938, he had been ill for about two years, and at the time of that operation the tumor was already very large and inoperable. It is therefore quite possible that the leukemoid state and the neoplastic growth may have developed simultaneously.

TABLE 1—*Blood Findings in Case P I P (Cancer of the Stomach)*

	Date							
	Sept. 24 35	Oct. 21 35	Jan. 14 36	March 10 36	April 3 37	April 21 38	May 5 38	Nov. 7 38
Hb%	80	89	91	88	106	49	52	66
RBC	3,65	4,18	4,20	4,60	4,64	3,72	3,92	3,69
WBC	64,000	36,000	10,750	5,050	4,600	6,150	5,120	12,800
Neutrophils	4,7	1,7	18	29	53,3	80	82,5	93
Eosinophils	0,3	2,3	9,5	7,5	10,3	1	1	0
Basophils	0	0	0	1	0	0	0	0
Lymphocytes	94,3	94,7	67	55,5	28,6	9,3	9,5	2,3
Lymphocytes, abs	60,352	34,092	7,203	2,802	1,320	571	487	295
Monocytes	0,7	1	5	6	7	9,3	6,5	4,3
Plasma cells	0	0	0	0	0,6	0	0,5	0,3
Blasts	0	0,3	0,5	1	0	0,3	0	0

The following case in many respects shows a similar development. Unfortunately there is no microscopic examination of the gastric tumor available, but it is reasonable to suppose that the patient had a non-leukemic malignant growth there.

E J (Radium Center 1330/47) electrician, born 1871. Past history without interest. For six months had pains in the epigastrium after meals, and occasional vomiting. In May 1947 referred to the Radium Center to be treated for carcinoma of the stomach. He had lost 3-4 Kg. in weight during the preceding six months. There had been no angina or fever in connection with his present illness. The patient was pale and emaciated. Numerous moderate enlargements of lymph nodes were present in the neck, axillae and groins, but no palpable enlargement of liver or spleen. In the epigastrium an irregular tumor was felt whose size was difficult to determine. There was no free acid in the stomach. Roentgen examination of the stomach showed a notched, irregular, eroded mass with a filling-defect for a distance of 8 or 9 cm. on the greater curvature. This was the size of an orange and extended into the lumen. On admission the sedimentation rate was 43 mm./hour, hemoglobin 77 per cent, red cells 3,680,000, white cells 11,600 with neutrophils 17, eosinophils 0 and mononuclears 83 per cent. Most of the mononuclears were of the small lymphocyte type with very scant cytoplasm and a nucleus rich in chromatin. But a few larger forms were also present with a monocytoïd configuration and structure, often with distinct nucleoli. A number of smudge cells were seen. Only a few typical monocytes were present. No McKinley cells. The sternal marrow was rich in cells and contained over 80 per cent of these atypical mononuclear cells. Microscopic examination of a lymph node puncture showed typical lymphatic leukemic changes. The Paul Bunnell test was negative. The patient was treated with x rays over the tumor of the stomach (rotatory irradiation) 160 kV., 9½ ma., through 0.5 mm. Cu + 1 mm. Al. half layer value 0.7, distance 50 cm., two

series each of 2,700 r. The treatment did not reduce the size of the epigastric tumor substantially, and the roentgenographs showed only moderate regression of the tumor in the stomach. The patient was controlled as an out patient but became feebler and lost weight. The leukemic blood picture disappeared. (June 2, 3,500 white cells 46 mononuclears, June 17 4,800 white cells 16 mononuclears and no atypical cells. The mononuclear cells could now easily be divided in 9 per cent typical monocytes and 7 per cent lymphocytes.) In June 1947, the sternal marrow showed almost normal conditions, with only 14 per cent lymphocytes in the smears. The swelling of the lymph nodes also gradually diminished and by August of the same year they were only of hazelnut size. During the following months the patient was readmitted for renewed treatment. The lymph nodes had become still smaller, but the tumor of the stomach had become larger. The blood counts were as follows: hemoglobin 45 per cent, red cells 2,100,000, white cells 6,400, platelets 4,000. Neutrophils 83.5, eosinophils, 0.5, lymphocytes 1.5, monocytes 6. No atypical cells. He was again given roentgen treatment this time to two fields, each of 5 x 10 cm, over the epigastrium 160 Kv 4 ma through 1 mm Cu + 1 mm Al distance 40 cm 600 r, in all to each field. During his stay he became steadily feebler and when discharged a month later he was rather cachectic. At the time of discharge there was no glandular swellings except a single bean-sized node on one side of the groin. The blood counts at this time (October 10) were: hemoglobin 62, red cells 3,230,000, white cells 5,400, neutrophils 88, lymphocytes 10, monocytes 2. As will be seen there was now both relative and absolute lymphopenia. The sternal marrow showed no evidence of leukemia. Three weeks later he died at home, and unfortunately we did not succeed in obtaining a necropsy.

*Comment.* This third case has some resemblance to the foregoing. On admission, there were clinical and hematologic signs of lymphatic leukemia with typical changes in the sternal marrow and the examined lymph node. At the same time, a tumor was found in the stomach, and as this grew larger all signs of leukosis disappeared,\* the swellings of the lymph nodes subsided, the sternal marrow became normal and at last there was marked lymphopenia in the blood. Unfortunately we were unable to examine the tumor of the stomach histologically, but it is hardly possible that it could have been a lymphatic leukemic growth, as these are extremely radiosensitive, while in this case there was very moderate regression in spite of intensive local irradiation with roentgen rays.

The coexistence of cancer and leukemia in the same individual has been observed rather often, and some of the reported cases of this association have recently been reviewed by Videback.<sup>24</sup> In the present study, it is specially the combination of cancer and *lymphatic* leukemia which is of interest. Such cases have been reported by Lannois and Regaud<sup>17</sup> (cancer of the uterine cervix), Marischler<sup>18</sup> (hypernephroma), Fuhs<sup>12</sup> (cancer of the skin), Genévrier<sup>13</sup> (pulmonary cancer), Scheufler<sup>19</sup> (cancer of the skin), Brückner<sup>5</sup> (cancer of the uterine cervix) Schreiner and Wehr<sup>20</sup> (cancer of the skin, 2 cases, pulmonary cancer, 1 case, mammary cancer, 1 case), Saupe<sup>28</sup> (cancer of the stomach), Denoyer<sup>9</sup> (cancer of the larynx), Pulvertaft<sup>24</sup> (cancer of the skin), Penzold<sup>22</sup> (cancer of the stomach), Askanazy<sup>1</sup> (cancer of the esophagus), Dustin<sup>10</sup> (cancer of the stomach), Engelbreth Holm<sup>11</sup> (cancer of the lip, 1 case, cancer of the skin, 5 cases, cancer of the penis, 1 case), Hertz<sup>15</sup> (cancer of the kidney), Švejška<sup>33</sup> (cancer of the stomach, 1 case, cancer of the larynx, 1 case) Gertler<sup>14</sup> (cancer of the skin), Ovnbyl and Therkildsen<sup>21</sup> (cancer of the breast and the prostate in the same individual), Delcourt (mammary cancer,<sup>7</sup> cancer of the bile ducts in the liver<sup>6</sup>), Morrison<sup>19</sup> (cancer of the pancreas), Berk and Movitt<sup>3</sup>

\* One must consider the possible effect on the leukemia of the local roentgen ray therapy directed to the stomach. *Editor*

(cancer of the larynx), Videback<sup>24</sup> (cancer of the skin) In this review, we have omitted the combination of tumors originating in the hemopoietic tissue (lymphosarcomas, reticulosarcomas, myelomas, etc.) with leukemic blood pictures

Petri<sup>25</sup> found carcinoids in the intestines of 2 patients with aleukemic lymphatic leukemia and called attention to the advisability of a close examination of the intestine of patients with leukemia, with the view of the possible presence of such tumors, which are often small and difficult to distinguish from Peyer's patches, especially if there is also leukemic infiltration in the gut

That leukemic reactions of the myeloid type may occur in connection with malignant tumors is well known, though the mechanism of their development is not quite clear Lymphatic reactions, on the other hand, are rarely seen in connection with malignant tumors (here we again omit the special tumors arising from the hemopoietic tissue) Reich<sup>26</sup> described a curious case of an adenocarcinoma of the sigmoid in a man 55 years old with 18,700 leukocytes per cu mm, 91 per cent of which were lymphocytes The sternal marrow showed marked infiltration of lymphocytes, many of which were abnormal The day before he died, the leukocyte count rose to 103,000 per cmm, with 95 per cent lymphocytes, but at necropsy there was no leukemia Reich suggests that the unusual hematologic picture may have been due to an action of the carcinoma on the hemopoietic tissue The necropsy revealed generalized metastases, including the bone marrow Müller and Wertheimann<sup>27</sup> mention a case of lymphocytosis associated with mammary cancer with metastases to spleen, lymph nodes and bone marrow There were 33,000 leukocytes per cmm, with 63 per cent lymphocytes At necropsy, no leukemic changes were found in the organs A case which is not quite clear, however, is reported from Russia, by Šal<sup>28</sup> The patient was a man, 55 years old, with 434,000 leukocytes per cu mm, 99 per cent of which were lymphocytes At necropsy, cancer of the peritoneum (? primary tumor) was found, with enlargement of the spleen and liver, but no enlargement of the lymph nodes The bone marrow was normal In the case of a woman 29 years old, reported by Winans,<sup>29</sup> blood examinations showed up to 18,500 leukocytes per cu mm with 85 per cent lymphocytes, but there was no enlargement either of the spleen or the lymph nodes Some time afterwards, she experienced pains in the lower part of the abdomen, and at the operation a pseudomucinoid cyst was removed from the right ovary, and a papillary adenoma from the left The lymphocytosis disappeared, but had already decreased before the operation and seemed to have been due to a febrile infection of the upper air passages and not to the tumors Rohr and Hegglin,<sup>30</sup> in their monograph on the occurrence of tumor cells in sternal punctures, briefly mention a case of a simple, solid carcinoma of the cardia in a man, 75 years old, with 25,000 leukocytes per cu mm, 34 per cent of which were lymphocytes Die Lymphocyten sind vorwiegend jung (Bild der lymphatischen Reaktion) In the sternal marrow, there were 17,600 per cent lymphocytes besides the tumor cells In Silberstern and Pechterewa's case<sup>31</sup> of a cancer of the rectum in a 72-year old male there were 18,000 leukocytes with 85 per cent lymphocytes, without other signs of leukemia The autopsy revealed metastases to the regional lymph nodes but no leukemic involvement of the organs and only slight lymphocytic infiltration of the liver and bone marrow

In comparison with the myeloid reactions in malignant tumors, lymphatic reactions are extremely rare. Moreover, they seem to be different from the myeloid in several respects, thus they, in contrast to these, which as a rule are a late phenomenon, often occur at a very early stage of the development of the cancer, sometimes even at the same time as the latter. The mechanism which elicits the lymphatic reactions in these cases is quite obscure. Silhol<sup>22</sup> thought that metastases from cancer of the stomach to regional lymph nodes might give rise to lymphocytosis, but the early occurrence of the lymphatic reactions makes it doubtful if metastases to lymph nodes play any part. Another strange thing about these reactions is, as far as one can conclude from a rather few cases, their tendency to become less pronounced as the malignant tumor grows larger. The same tendency can be noticed as regards the lymphatic leukemias associated with cancer, for instance, in Marischler's<sup>18</sup> case of hypernephroma combined with chronic lymphatic leukemia, and in the first of the cases reported in the present communication. In Marischler's case, the number of lymphocytes steadily decreased, and the lymph nodes gradually became smaller as the disease developed. He supposed that this was due to an effect of cancer toxins on the leukemic process, similar to the well-known effect of certain infections. It must be remembered, however, that the lymphatic tissue is apt to be strongly affected by cachexia and inanition, conditions which appear with malignant tumors, especially when these have their origin in the gastrointestinal tract.

Even in the uncomplicated cases of chronic lymphatic leukemia there is often a fall in the lymphocyte content of the blood during the last days of life, and the spleen and lymph nodes may become smaller, even without therapy, though, of course, this does not mean that there is an entire disappearance of the leukemic changes in the organs. Of course it is a question if certain malignant tumors of the gastrointestinal tract do not have a repressing effect on the leukemic processes in the organs. That tumors of the stomach do not always have this effect, is clearly seen from Penzold's case, in which the neoplastic growth and the leukemic processes existed side by side and were even believed by Penzold to have activated each other. It is clear that as basis for judgment respecting the reciprocal effect of the tumor and the leukemia only those cases can be used in which the observation time has been sufficiently long, and in which the patient died either of the leukemia or of the malignant tumor, and not of some irrelevant disease. In Dustin's case,<sup>10</sup> for instance, of stomach cancer with lymphatic leukemia, the patient was only observed a few days and died of an intercurrent infection.

#### SUMMARY

The author reports the case of a patient with chronic lymphatic leukemia, who after some years developed dyspeptic symptoms, increasing cachexia, and eventually died. The leukemia had been subleukemic for several years. Necropsy revealed an adenocarcinoma of the pylorus and lymphatic leukemic changes in the lymph nodes, spleen and liver. In two other cases a lymphatic leukemic blood picture and clinical signs of leukemia (including lymph node enlargements and leukemic changes in the bone marrow) gradually disappeared as tumors of the stomach de-

veloped, and in both cases the leukemic blood picture was replaced by a state of lymphopenia. In one of them, the necropsy revealed an adenocarcinoma of the pylorus, in the other, necropsy could not be obtained, but the clinical picture and the radiosopic examinations strongly suggested carcinoma of the stomach in this case, too. These last two cases must be interpreted as lymphatic leukemoid states produced by the presence of the carcinomatous neoplasms, though the possibility can not be excluded that certain carcinomas of the gastrointestinal tract may be capable of primarily or secondarily exercising an inhibitory influence on the leukemic processes.

In connection with the report of these cases, the author reviews the cases from the literature, of lymphatic reactions in cancer and of the coexistence of lymphatic leukemia and cancer in the same individual.

## REFERENCES

- <sup>1</sup> ASKANAZY M. Leukämie und Tumoren. *Schw Med Wochenschr* 21: 29-34 1940.
- <sup>2</sup> BECLERE AND H. BECLÈRE. Die radiotherapeutische Behandlung der Leukämie. *Strahlenther* 3: 553-560 1913.
- <sup>3</sup> BERE M. AND MOVITT E. R. Leukemia complicated by cancer. *Am J Clin Path* 15: 246-249 1945.
- <sup>4</sup> BICHEL, J. Lymphatic leukosis influenced by carcinoma of the stomach. *Fol haem* 64: 91-96 1940.
- <sup>5</sup> BRÜCKNER. Lymphatische Leukämie und Portiocarcinom. *Arch f Gynäkologie* 157: 616-619 1934.
- <sup>6</sup> BULO K. M. Ein mit Radiothor behandelter Fall von myeloischer Leukämie mit komplizierendem Magenkarzinom. *Deutsch Med Wchnschr* 56: 881-882 1924.
- <sup>7</sup> DELCOURT R. Les tumeurs doubles à tissus histologiquement différents. *Bull Cancer* 31: 128-142, 1943.
- <sup>8</sup> — AND VAN VLIET P. Coexistence d'une leucémie lymphoïde et d'un carcinome. *Acta Med Scand* 119: 47-56 1944.
- <sup>9</sup> DENOTER A. Carcinoma laryngeum in leucemico. *Ann di laring otol* 36: 185-192 1936.
- <sup>10</sup> DUSTIN P. Coexistence d'une leucémie lymphoïde et d'un carcinome gastrique. *Revue belge d Sci ences Med* 13: 199-208 1941.
- <sup>11</sup> ENOELBRETH HOLM J. Leukæmi og anden malign Svulst hos samme Patient. *Nordisk Medicin* 9: 791-799 1941.
- <sup>12</sup> FURS. Multizentrische Basalzellenepitheliome bei lymphatischer Leukämie. *Dermat Wchnschr* 85: 1533-1533 1927.
- <sup>13</sup> GENEVRIER LORRAIN AND COIRRE. Lymphadénome leucémique associé à un épithélioma du poulmon. *Arch Médico-Chir de l Appareil Resp* 5: 195-104 1930.
- <sup>14</sup> GERTLER. Lymphatische Leukämie bei gleichzeitigen Haut-Carcinomen. *Zentralb f Haut und Gesch lechtskrankh* 68: 271-271 1942.
- <sup>15</sup> HOTZ A. Zur Differentialdiagnose Agranulocytose Leukämie. *Zeitschr f Kinderheilk* 62: 529-540 1941.
- <sup>16</sup> KAST H. W. Leukämioide Reaktionen bei malignen Tumoren. *Deutsch Arch f Klin Med* 188: 173-180 1942.
- <sup>17</sup> LANNOS M. AND REAUD C. Coexistence de la leucocythémie vraie et d'un cancer épithélial. *Arch de Med Exp et d Anat Path* 7: 254-264 1895.
- <sup>18</sup> MARISCHLER J. Ein Fall von lymphatischer Leukämie und einem Grawitz'schen Tumor der rechten Niere. *Wien Klin Wchnschr* 9: 686-690 1896.
- <sup>19</sup> MORRISON M. FELDMAN F. AND SAMWICK A. A. Carcinoma and leukemia. Report of two cases with combined lesions. review of literature. *Ann Int Med* 5: 75-84 1944.
- <sup>20</sup> MÜLLER A. AND WERTHEIMANN A. Unter dem Bilde der sogen. Leukämie verlaufende Karzinose des Knochenmarkes bei kleinem verstecktem Mammakarzinom. *Fol haem* 46: 4-9-439 1932.



- <sup>21</sup> OVNBØLL, A. AND TERKILDSEN, K. Et Tilfælde af Sarcom Carcinom og Leukose hos samme Patient. *Nordisk Medicin* 20 1662-1662, 1943
- <sup>22</sup> PENZOLD H. Leukämie und Carcinom. *Deutsch Arch klin Med.* 180 430-438 1937
- <sup>23</sup> PETRI, S. Undersøgelser over de ved lymfatiske Leukose forekommende Celleformer (Polymorfi) og Mitoser samt Bidrag til Belysning af Sygdommens Stilling i Systemet. *Bibl f Læger* 120 153-316, 1928
- <sup>24</sup> PULVERTAFT, R. J. V. Multiple primary epithelioma in lymphatic leukaemia. *Brit. J Surg* 24 50-54, 1936
- <sup>25</sup> REICH C. A case of adenocarcinoma of the sigmoid with lymphatic leukemia blood picture. *Am. J Cancer* 26 781-782, 1936
- <sup>26</sup> ROHR K. AND HEGGLIN R. Tumorzellen im Sternalpunktat Metastasennachweis malignen Geschwülste im Knochenmark. *Deutsch Arch f klin Med* 179 61-79 1937
- <sup>27</sup> ŠAL, G. F. Lymphatic leukemic blood picture but pathoanatomical diagnosis of neoplasm (Russian). *Med Klin* 11 1149-1152, 1933
- <sup>28</sup> SAUPE E. Bestrahlungswirkung bei gleichzeitiger Erkrankung an leukämischer Lymphadenose und metastasierendem Magenkarzinom. *Wien klin Wochenschr* 49 1104-1105, 1936
- <sup>29</sup> SCHEUFFLER, A. Carcinombildung auf einem Leukämied. *Arch f Dermat. und Syph.* 161 586-596, 1933
- <sup>30</sup> SCHREINER, B. F., AND WEHR, W. H. Cancer associated with leukemia. *Am. J Cancer* 21 368-371, 1934
- <sup>31</sup> SILBERSTEIN E. AND PECHTEREWA, L. N. Ein Beitrag zur Frage der lymphatischen Reaktion (Lymphatisches Blutbild bei einem Falle von Mastdarmkrebs). *Wien Arch f inn Med.* 29 47-66 1930
- <sup>32</sup> SILHOL, J. L. Examen du sang en chirurgie. *Revue de Chir* 21 (1) 734-742, 1901
- <sup>33</sup> SVEJDA, J. Two cases of lymphatic leukemia and carcinoma. (Tjekkisk) *Čas Lék. česk.* 1942 p. 1372-1373
- <sup>34</sup> VIGORBAEK, A. Heredity in human leukemia and its relation to cancer. *Diss. Copenhagen* 1947
- <sup>35</sup> WINANS, H. M. Leukemoid blood picture in pseudomucinous cyst and papillary adenoma of the ovary. *Am J M. Sc.* 181 251-254 1931
- <sup>36</sup> ZADNEK, I. Radiothorium bei leukämischer Myelose 1. *Fol haem* 49 287-340, 1933

# LYMPHOCYTIC LEUKEMOID REACTION OF THE BLOOD ASSOCIATED WITH MILIARY TUBERCULOSIS

By FRANK H. GARDNER, M D , AND STACY R. METTIER, M D

BLOOD pictures similar to those of myelocytic and lymphocytic leukemia have been reported<sup>1-6</sup> to occur not infrequently in patients with miliary tuberculosis. These reports indicate that there is a marked 'shift to the left' of the leukocytes in the peripheral blood to include varying percentages of myelocytes and myeloblasts. This hemogram is associated with an absence of the characteristic leukemic infiltration of the tissues on postmortem examination.

Landon<sup>7</sup> has reported a case of tuberculous bronchopneumonia in a 16 year old girl in whom the white blood cell count rose to 36,800 per cubic millimeter of blood, 95 per cent of the cells were considered to be immature lymphocytes. Coley and Ewing<sup>8</sup> reported the case history of a 42 year old woman with diffuse tuberculosis of the lymph nodes. The white blood cell count was 8,000 cells per cubic millimeter of blood, of which 84 per cent were reported as of the mononuclear type. The mononuclear cells were considered to be lymphocytes. The structural changes in the lymph nodes showed acute necrosis without caseation and without tubercle formation.

Leibowitz<sup>9</sup> has reported the occurrence of a predominantly myeloblastic blood picture in a patient with symptoms of sepsis associated with miliary tuberculosis. Examination of tissues removed from various organs showed necrotic lesions containing myriads of tubercle bacilli but without tubercle formation.

One case with a monocytic leukemoid reaction has been studied.<sup>10</sup> The patient showed a white blood count of 82,000 cells per cubic millimeter of which 42 per cent were monocytes. Autopsy revealed generalized tuberculous adenitis. It was of interest that the monocytes were found only in association with the tuberculous foci in the lung and liver. The author suggested that the monocytic response might be due to a reactive irritation of the reticulo-endothelial system.

The following two case histories are reported as examples of a lymphocytic leukemoid response to miliary tuberculosis.

## CASE REPORTS

### CASE I\*

R. O., a 59 year old white female, entered the Mt. Zion Hospital on January 5, 1945. She complained of generalized malaise and a swelling in the left side of her neck. Four months previously the patient had noted that she tired easily and had lost 20 pounds in weight in one month's time. Profuse perspiration at night caused her considerable discomfort. She was aware of dizziness upon sudden change of position. Just prior to entry it had been observed that she was febrile.

The patient stated that a nodular mass had been removed surgically from the left side of her neck at the age of 16. This consisted of one walnut-sized node surrounded by many smaller nodules. In 1938

From the Division of Medicine, University of California Medical School, San Francisco, California.  
Aided by a grant from the Edward S. James Foundation.

\* We wish to thank Dr. Roy Morris for permission to use this case history.

at the age of 53 the patient again entered the hospital because of the appearance of a mass in the left side of the neck which extended from the mastoid process to the angle of the mandible. The mass was firm in consistency, smooth in outline, and fixed to the underlying structures. Six exposures to roentgen ray did not alter its size, and it was surgically removed. The pathologist reported atypical tuberculosis, and acid fast organisms were found in the stained sections. In January 1943 she saw her physician because serous fluid drained from the area of the wound in the left side of the neck, where the nodes had been excised. With symptomatic treatment this sinus healed in two months. Nine months later she was seen again because of climacteric symptoms. At this time the wound was healed and she had gained weight. She did not see her physician again until the present illness.

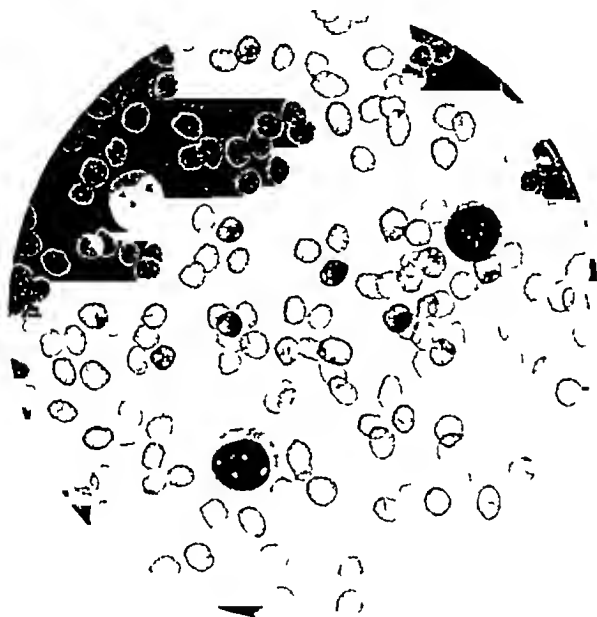


FIG. 1—CASE 1 ( $\times 1000$ ) Film prepared from peripheral blood and stained according to Wright's technic. Note the immature lymphocytes.

Physical examination revealed a well-nourished woman in no apparent distress. Blood pressure was 116/70 mm Hg, pulse 88. There was a large, matted, movable mass in the region of the left submental triangle. Small nodes were palpable bilaterally in the anterior and posterior cervical chains. The heart was not enlarged and there were no cardiac murmurs. No abnormal findings were apparent on physical examination of the chest. The tip of the spleen was palpable 4 cm. below the left midcostal margin. The rest of the physical examination was essentially negative. No other adenopathy was noted.

Urinalysis revealed 1+ albumin, specific gravity of 1.019, no sediment abnormalities on microscopic examination. Blood examination showed hemoglobin of 8.9 Gm. or 58.4 per cent (Sahli); the red blood cell count was 3.02 million per cubic millimeter of blood; the white blood cell count was 6,100 per cubic millimeter of blood. The differential count showed lymphoblasts 11 per cent, prolymphocytes 64 per cent, lymphocytes 24 per cent, granulocytes 1 per cent. Almost all of the cells showed characteristics of young lymphocytes and lymphoblasts. The cytoplasm was deeply basophilic and contained a moderate number of azure granules. The chromatin was diffuse and between its meshes could be discerned several large nucleoli (figure 1). Differential counts done on four different occasions between January 6 and January 29, 1945, showed results similar to the first count.

In addition to symptomatic treatment the patient received four blood transfusions over a period of two weeks with no subjective or objective improvement. The patient maintained a swinging daily temperature curve with peaks at 39.5 C and 40.5 C. The fever followed no specific pattern but for the most part was above 38 C. About February 4, 1945 she began to have periods of disorientation. On February 11, her respirations were labored and the patient became comatose and expired twenty nine days after entry into the hospital.

**Clinical diagnosis:** Tuberculous adenitis, acute lymphocytic leukemia, aleukemic myelophthisic anemia.

### *Necropsy*

**Gross Examination:** The body was that of an obese white woman with generalized icterus. Multiple petechiae were present over the body and about both eyes. No palpable subcutaneous lymph nodes were noted. There was no excess free fluid in any of the body cavities. The organs were normally disposed.

The heart weighed 320 grams and was of usual contour and quite flabby. It was yellow-tan, striated and soft. The coronary ostia and the major coronary branches were patent.

The weight of the left lung was 620 grams, the right 410 grams. The pleural surfaces were smooth, but on cutting were crepitant and dark red. The trachea and bronchi contained a moderate amount of frothy hemorrhagic material. The tracheobronchial lymph nodes were large, measuring up to 4 cm. They were grayish white when sectioned.

The liver weighed 1900 grams and was quite soft. The capsule was smooth and on the cut surface the parenchyma was yellow and finely dotted with red. The gallbladder, pancreas and adrenal glands showed no gross changes.

The gastrointestinal tract showed no gross changes. Near the cecum there was a mass of translucent tissue resembling matted lymph nodes which measured 6 cm. in diameter. On section this was grayish white with yellow foci.

The spleen weighed 440 grams and was soft. On the cut surface it was dark red dotted with gray.

The combined weight of the kidneys was 380 grams. On cut surfaces they showed multiple hemorrhagic markings but otherwise were not abnormal. The right ovary was replaced by a cystic mass filled with thick hemorrhagic material. Otherwise the pelvic organs were normal. There were no additional gross abnormalities of significance.

### *Microscopic Examination*

**Heart:** The myofibrillae were thin with prominent striations and nuclei. The small vessel walls showed no changes.

**Lungs:** The alveoli were collapsed in large areas and the small vessels were distended. Elsewhere the alveoli and bronchioles contained granular eosinophilic material and a few polymorphonuclear cells.

**Liver:** The architecture was distorted by atrophy and the presence of broken-down cells in the central areas. There were many oval areas of necrotic tissue with an average diameter of one third of a lobule. These areas were scattered throughout the liver and were composed of dense eosinophilic amorphous necrotic tissue in which a few ghosted nuclei were seen. There was a fine border of scattered lymphocytes about some of the nodules. Silver stain showed the usual reticulum network intact except in the caseous areas. Sections of liver stained by the Ziehl-Nielsen technique revealed numerous clumps of acid-fast bacilli. No periportal lymphocytic infiltration was seen.

**Spleen:** The lymph follicles were quite small and sharply bounded by congested red pulp. Throughout the organ were necrotic foci of the same size as those observed in the liver and of similar appearance. Acid-fast organisms were noted in these caseous nodules also.

**Lymph nodes:** Sections of the lymph nodes from the cervical, tracheo-bronchial, pre-aortic and mesenteric groups showed the same picture of numerous eosinophilic oval areas of necrosis. Again they were devoid of cells, blending peripherally with lymph node structures. These areas were devoid of reticulum by silver stain and contained myriads of acid-fast organisms.

**Bone marrow:** Spread diffusely throughout the marrow were numerous areas of necrosis which were devoid of any epithelioid reaction at their borders. The blood-formative tissue was slightly hypoplastic. Megakaryocytes were rarely seen but the plasma cells were slightly increased.

**Miscellaneous:** Sections of the adrenal gland, pancreas, parathyroid, thyroid, uterus, ovaries, kidney,

gallbladder, and urinary bladder showed no changes of significance. No changes were found in the brain or meninges.

**Anatomic Diagnosis** Generalized tuberculosis of lymph nodes - (a) miliary tuberculosis (b) hypoplasia of bone marrow hemorrhagic cyst of ovary

#### CASE 2

V. L., U130787, a 73 year old man, entered the University of California Hospital on August 19, 1946, complaining of dyspnea, orthopnea, and hemoptysis. The patient's history dated back to 1918 when he had his first episode of hemoptysis, which was treated with three weeks of bed rest. Again in 1923 he had an episode of severe hemoptysis and was told at that time that he had pulmonary tuberculosis. Otherwise the past history was noncontributory. In October, 1945, the patient had a swelling of the right ankle and lower leg and a diagnosis of phlebitis was made. In February, 1946, he saw his physician because of generalized malaise. A white blood cell count at that time revealed a leukocytosis of 66,000 cells per cubic millimeter, with 95 per cent lymphocytes. There was no hepatosplenomegaly or adenopathy noted. He was given symptomatic therapy until June 18, 1946. At that time he was given Fowler's solution, 5 drops three times daily. However, the patient stopped the medication in five days because of nausea. The drug was again started and continued for the first two weeks of July. The white blood cell count averaged about 39,000 cells per cubic millimeter with 90 per cent lymphocytes at this time. During the two months preceding hospitalization, the patient had a cough productive of blood tinged sputum. He also suffered from night sweats and fever.

On entry the patient was dyspneic and cyanotic. Blood pressure was 125 systolic and 70 diastolic. The temperature was 38.2 C, the pulse 120 per minute, the respirations 30 per minute. On physical examination no adenopathy was noted. The trachea was deviated to the right and there was marked venous distention of the neck. The chest showed atrophy of the right shoulder girdle muscles. There was limited excursion of the right chest. There was flatness of the right upper third of the chest posteriorly and anteriorly to percussion. Crepitant rales were present over the entire chest with bronchial breathing over the right apex. No cardiac enlargement was noted. On deep inspiration the liver was palpable 7 cm. below the right midcostal margin and the spleen 3 cm. below the left midcostal margin. Bilateral pedal edema was present with brownish discoloration over the right ankle.

#### Laboratory Data

The urine showed faint albuminuria and had a specific gravity of 1.026. There were no abnormal findings in the sediment. Examination of the blood revealed hemoglobin of 9.5 grams or 66 per cent (Sahli). The red blood cell count was 3.5 million per cubic millimeter, white blood cell count was 42,500 cells per cubic millimeter, the differential count showed prolymphocytes 6 per cent, lymphocytes 77 per cent, degenerative cells 13 per cent, granulocytes 4 per cent. An adequate number of platelets were present on blood films prepared with Wright's stain. The sputum contained large numbers of acid fast organisms. The electrocardiogram showed an abnormal record suggesting coronary artery disease.

The patient was immediately digitalized with 8 cc. of Cedilanid intravenously and soon obtained marked relief from dyspnea. He was then given a maintenance dose of digitalis folia, 0.1 Gm. twice daily. The cyanosis receded slowly but the patient continued to have a fever of between 38 and 39 C. at all times. A chest x-ray taken shortly after entry revealed extensive infiltration of the upper lobes bilaterally with marked pulmonary shrinkage on the right side displacing the mediastinal structures. A homogeneously distributed nodular peribronchial infiltration was present throughout both lungs.

It was felt that the patient could receive convalescent care at home and he was discharged eleven days after entry. Before discharge he was given 500 cc. of citrated blood which was well tolerated. At time of discharge his white blood cell count was 73,600 per cubic millimeter with 70 per cent lymphocytes and the red cell count was 4.12 million per cubic millimeter. A roentgenogram of the chest taken on the day of discharge showed a marked decrease in the transverse diameter of the heart from 13 cm. to 10.3 cm.

The patient was confined to bed at home. He was orthopneic and continued to have a productive cough and septic fever. He died September 14, 1946, seventeen days after discharge from the hospital.

**Clinical diagnosis** miliary tuberculosis far advanced pulmonary tuberculosis lymphocytic leukemia arteriosclerotic heart disease

*Necropsy*

Gross examination revealed the body of an emaciated male. No lymph nodes were palpable. The right pleural cavity was obliterated because of adhesions. The trachea was deviated to the right and the lymph nodes of the mediastinum showed enlargement and pigmentation. The heart weighed 320 grams and the coronary vessels were patent throughout.

The right lung weighed 890 grams, the left 1010 grams. On the cut surface they revealed marked fibrosis with grayish white infiltrations 1 to 3 mm in diameter throughout the parenchyma. The right upper lobe revealed a small cavity 1 cm in diameter.

The spleen weighed 300 grams. On section the corpuses were well defined, but there were diffuse gray infiltrations throughout the pulp measuring up to 3 mm in diameter.

The liver weighed 1830 grams and on section showed occasional whitish areas among the otherwise normal parenchyma. The kidneys were of normal size and architecture. Numerous pinhead gray areas were spread throughout the cortex. The same type of infiltration was noted in the sections of the adrenals.

The abdominal and mesenteric lymph nodes were enlarged. The bone marrow was pale but not remarkable otherwise. No other gross abnormal findings were noted.

*Microscopic Examination*

**Lungs.** The bronchi and bronchioles were dilated and showed peribronchial fibrous proliferation. An occasional conglomerate tubercle with central caseation and surrounding fibrous reaction was noted. Within a dilated vascular channel a mass of tuberculous granulation tissue was seen and suggested a possible source of the miliary spread. In the alveoli surrounding the early conglomerate masses of tubercles proliferating fibroblasts and epithelioid cells were seen. In addition to the older process, there was a widespread distribution of single or multiple young tubercles with little surrounding fibrous reaction.

**Spleen.** The parenchyma was largely replaced by tubercles showing minimal central necrosis and containing giant cells. In the scanty uninvolved areas there was no obliteration of the sinusoids which contained many lymphocytes, large mononuclear cells, and a few red blood cells. A moderate epithelioid hyperplasia was noted. The rare germinal centers were distorted and replaced in part by young and old lymphocytes and many mononuclear cells.

**Liver.** The hepatic lobular pattern was well maintained. The sinusoids were distended but contained few cells. These were chiefly red blood cells. There were few lymphocytes, monocytes, or polymorphonuclear cells. Throughout the parenchyma young and old tubercles could be discerned. These contained giant cells and showed slight necrosis. Immediately adjacent to the tubercles, particularly in the periportal connective tissue, were large numbers of mature lymphocytes.

**Kidneys.** Several areas contained tubercles with central necrosis. Other areas showed large conglomerate portions of tissue with widespread caseation and a marked lymphocytic infiltration at the border. The intervening glomeruli and tubules appeared normal.

**Lymph nodes.** Bronchial and mesenteric nodes showed preservation of the normal architecture with a marked hyperplasia of the reticulo-endothelial pattern. The sinusoids were intact and contained many lymphocytes and mononuclear and plasma cells. Tubercles were widely scattered among the intact lymphoid follicles. These were usually small without caseation but showed marked giant cell formation. No capsular invasion nor abnormal number of mitoses was seen (figure 2).

**Bone marrow.** Several sections of sternal and vertebral marrow revealed extensive single and conglomerate tubercle formation with marked caseation and trabecular bone destruction. Aside from the tubercle formation there was a normal quantitative and qualitative relationship of the myelopoietic and erythropoietic series. Megakaryocytes were present in adequate numbers (figure 3).

**Miscellaneous.** Sections of the adrenals showed diffuse tubercle formation in the cortex and medulla with dense fibrous replacement. Studies of the thyroid, pancreas, gallbladder, testes, and prostate showed no changes of significance.

**Anatomic diagnosis.** (1) Bilateral pulmonary tuberculosis, fibrocaceous type, with cavity of right apex and diffuse right pleural adhesions. (2) Miliary tuberculosis of lungs (bilateral), liver, spleen, adrenals, lymph nodes, and bone marrow. (3) Reactive lymphoid hyperplasia of lymph nodes. (4) Lymphocytic leukemoid reaction of bone marrow. (5) Generalized arteriosclerosis with moderate coronary sclerosis and focal myocardial fibrosis.

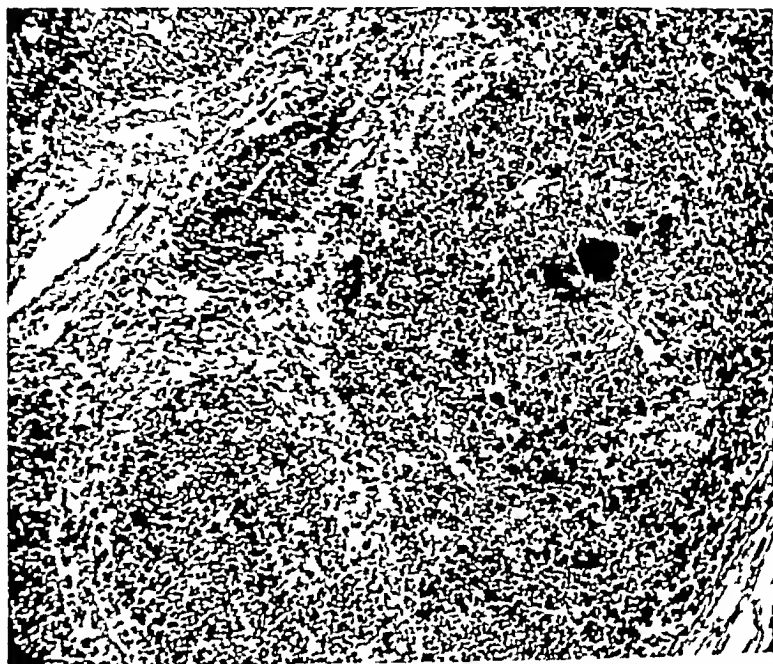


FIG 2—CASE 2, LYMPH NODE ( $\times 120$ ) Typical section of the diffuse tuberculous invasion. Diffuse hyperplasia with intact capsular wall present (lower right corner)



FIG 3—CASE 2, BONE MARROW ( $\times 120$ ) The epithelial process is surrounded by marrow of normal architecture

## DISCUSSION

Both of these cases were diagnosed as lymphocytic leukemia when first seen by members of the Hematology Unit. It was not until necropsy that the question of a leukemoid response to tuberculosis arose. Krumbhar<sup>11</sup> has stated that it may not be possible to distinguish between a terminal leukemoid blood picture and a true leukemia. Such was true in these case studies.

In Case 1, the miliary tubercles showed a fine necrotic matrix with no proliferation of fibrous tissue. The entire process consisted of massive necrosis and the diagnosis of miliary tuberculosis was made by demonstrating acid-fast organisms in these areas. These lesions were similar to those described by Leibowitz<sup>9</sup> and Coley and Ewing.<sup>8</sup>

The report by Leibowitz<sup>9</sup> includes a review of the literature (especially European) of the necrotic lesions in tuberculous sepsis. One case reported by Marzullo and DeVeer<sup>4</sup> revealed no epithelioid changes at autopsy, but rather necrosis. On entry to the hospital this patient had a white blood cell count of 57,000 with 15 per cent myeloblasts. He died of tuberculous pneumonia.

Rich and McCordock<sup>12</sup> observed in animal experiments some correlation between the number of organisms and the extent of necrosis. The presence of extensive necrosis is probably correlated with the number of bacilli present. Upon reviewing their cases, these authors noted that acid-fast organisms were more numerous in the soft tubercles with extensive necrosis. In the more proliferative tubercle, the organisms were sparse. The soft tubercle is probably the result of a massive infection of the blood stream associated with a high degree of allergy.

Such a condition probably existed in Case 1. A long-standing tuberculous infection in the neck was associated with a miliary sepsis. Can we consider the lymphocytic leukemoid picture to be an agonal response to the infection? The interesting study of Wiseman and Doan<sup>13</sup> may aid in understanding the lymphocytic response. These authors showed that the age of the lymphocyte can be determined by progressive variations in cellular cytology, namely, basophilia of the cytoplasm, chromatic density, and distribution of the non-segmented nucleus. They divided the circulatory lymphocytes into three classes—young, mature, and old cells. It was observed in rabbits that there was a marked increase in the percentage of young lymphocytes following infection by intravenous injection of avian tuberculosis bacilli. As the animal neared death from miliary tuberculosis, there was a sharp decline in the percentage of mature forms. Studies of clinical material also showed an increase in young lymphocytes with progression of pulmonary tuberculosis. The authors felt that the increase in young lymphocytes with tuberculosis infection indicated that these blood elements were utilized in the pathologic process.

From this study can we postulate that a marked stimulus of the tuberculotoxins shifted the response of the lymphocyte to the early stem forms? This might help explain the genesis of the lymphocytic leukemoid blood picture in the first case.

Case 2 presented a more controversial problem. In a patient of this age, with an illness of long duration and an elevated white blood cell count, the diagnosis of chronic lymphocytic leukemia was more tenable. It was believed when this patient was first studied that a chronic leukemia had activated an old fibrotic tuberculosis



and that this was associated then with a diffuse hematogenous peribronchial extension culminating in cardiac embarrassment from a subacute cor pulmonale. The tender hepatomegaly and splenomegaly were first noted when the patient entered the hospital with cardiac embarrassment. This patient had been observed by a staff hematologist regularly for five months before entry, and at no time did he note adenopathy or hepatosplenomegaly.

Rossle<sup>14</sup> has observed patients with lymphocytic leukemia without adenopathy or hepatosplenomegaly. However, microscopic examination of the bone marrow in these cases revealed leukemic infiltration. In the second of our cases, necropsy findings of young and old tubercles throughout the organs and lymph nodes suggest a repeated bacteremia. Can we postulate that this patient had a persistent leukemoid reaction for six months before death? In a study of leukemoid reactions, Hill and Duncan<sup>15</sup> recently reported a case of leukemoid reaction which existed over a three year period in a 40 year old Negro male who had been followed in a leucic clinic. The white blood cell count varied from 23,000 to 78,400 per cubic millimeter of blood, and of these, 3 per cent were myeloblasts and 25 per cent myelocytes. Autopsy revealed a suppurative osteomyelitis of the sacrum associated with a gangrenous, necrotic abscess of the right thigh and an abscess of the right posterior lung field. In a similar manner, a persistent lymphocytosis might allow us to explain the blood findings in this case as a result of persistent irritation of the lymphoid tissue and marrow. Feldman and Stasney<sup>16</sup> have suggested an allergic response of the bone marrow to explain the myelocytic leukemoid blood response in tuberculous rabbits receiving tuberculin injections. We know of no experimental work showing lymphatic leukemoid response to tuberculin to indicate that lymphocytosis to the extent observed in these patients may be an allergic response to miliary tuberculosis. However, the lack of any evidence on microscopic examination of leukemic infiltration in the tissues in Case 2 forced us to conclude that the elevated white blood cell count of mature lymphocytes was the response to a progressive miliary tuberculosis.

Muller<sup>1</sup> has commented on the rarity of the leukemoid reaction. During a five year period in which approximately 2000 patients with tuberculosis were observed, no leukemoid blood pictures were seen. In rare cases, a few myelocytes were seen, and no case showed over 3 per cent myelocytes.

#### SUMMARY

Two cases of miliary tuberculosis that were diagnosed clinically as lymphocytic leukemia are presented. Both cases had evidence of chronic tuberculosis which was of 43 years' duration in Case 1 and of 28 years' duration in Case 2. Both patients had granulocytopenia and anemia.

Autopsy findings revealed no evidence of leukemic infiltration, but a diffuse miliary tuberculosis, involving all of the hematopoietic tissues, existed in both cases.

#### REFERENCES

1. MULLER, G. L. *Clinical Significance of the Blood in Tuberculosis*. New York, The Commonwealth Fund, 1943.

- <sup>1</sup> FOXNER, C. E. *Leukemia and Allied Conditions*. New York, Macmillan Company, 1938. P. 187.
- <sup>2</sup> CUSTER, R. P., AND CROCKER, W. J. The myeloleukemoid blood picture associated with tuberculosis. *Folia Haemat* 46: 359, 1932.
- <sup>3</sup> MARZULLO, E. R., AND DEVEER, J. A. Tuberculosis simulating acute leukemia. *Am J M Sc* 182: 372, 1931.
- <sup>4</sup> MILLS, E. S., AND TOWNSEND, S. R. Leukaemoid blood picture in tuberculosis. *Canad M A J* 37: 56, July 1937.
- <sup>5</sup> WEICHMANN, E. Miliartuberkulose und sekundäre Myeloblastose. *Med Klin* 18: 1086, 1922.
- <sup>6</sup> LANDON, J. F. Conditions simulating acute lymphatic leukemia. *Am J M Sc* 170: 37, 1925.
- <sup>7</sup> COLEY, W. B., AND EWING, J. Acute lymphatic tuberculosis with hemorrhagic purpura. *Proc N Y Path Soc.* 10: 145, 1910.
- <sup>8</sup> ——— AND ———. Acute lymphatic tuberculosis with hemorrhagic purpura. *Tr A Am Phys* 26: 175, 1911.
- <sup>9</sup> LEIBOWITZ, S. Tuberculous sepsis with a myeloblastic blood picture. *Arch Path* 25: 365, 1938.
- <sup>10</sup> GIBSON, A. Monocytic leukemoid reaction associated with tuberculosis and a mediastinal teratoma. *J Path & Bact* 58: 469, 1946.
- <sup>11</sup> KRUMHAR, E. B. Leukemoid blood picture in various clinical conditions. *Am J M Sc* 172: 519, 1926.
- <sup>12</sup> RICH, A. R., AND MCCURDOCK, H. H. An enquiry concerning the role of allergy, immunity, and other functions of importance in the pathogenesis of tuberculosis. *Bull Johns Hopkins Hosp* 44: 273, 1929.
- <sup>13</sup> WISEMAN, B. K., AND DOAN, C. A. The lymphatic reaction in tuberculosis. *Am Rev Tuberculosis* 38: 33, 1934.
- <sup>14</sup> ROSSLE, R. Lymphatische Leukämien ohne Systemerkrankung der Lymphknoten. *Virchow Archiv* 275: 310, 1930.
- <sup>15</sup> HILL, J. M., AND DUNCAN, C. N. Leukemoid reactions. *Am J M Sc* 201: 847, 1941.
- <sup>16</sup> FELDMAN, W. H., AND STASNEY, J. Leukemoid response in tuberculous rabbits to administration of tuberculin. *Am J M. Sc.* 193: 28, 1937.

## EDITORIAL

### WHAT'S IN A NAME

SEVERAL statements have appeared recently<sup>1 2 3</sup> concerning standardization of hematologic nomenclature. Their purpose is to bring clarity where there is confusion, uniformity where there is variety and order out of chaos. It is proposed that no new ideas be introduced but that terms be accepted which everyone will use in an agreed-upon sense, and that tables be made available in which a list of corresponding terms which are to be outmoded will be presented.

These statements reflect the considered views and sincere efforts of a number of individuals, to whom due credit must be given. The devotion of the chairman of the committee on nomenclature, which is the source of these reports, certainly deserves respect. It must be pointed out, however, that, although the published material gives the impression that the proposed nomenclature is desirable and widely supported, the contrary opinion is substantial and significant but has mainly found expression in informal conversations of persons interested in the field.

It can be admitted that hematologic terminology is confusing. However, there is reasonable doubt that the new proposals will improve matters. What is to be achieved by naming the stage of leukocyte preceding the myelocyte a progranulocyte when most people understand quite clearly what is meant by the term, promyelocyte? Will an official stamp have value by affixing an erroneous interpretation to the stab cell? Does a student gain a better understanding of physiology by being forced to call something a cell which is not one, such as the red corpuscle and the platelet, for which the terms erythrocyte and thrombocyte, respectively, are proposed? True, these terms are in current, though erroneous, use. But why endorse errors with a stamp of approval?

These, however, are comparatively minor criticisms of the proposed nomenclature. The recommended terminology for the red cell series impresses one as being artificial to an extreme. In an attempt to use corresponding classifications for the leukocytic and erythrocytic series of cells, differentiation is centered about nuclear rather than cytoplasmic features. As a consequence, a prorubricyte stage is introduced which can scarcely be differentiated, if at all, from the rubriblast. The concept of distinguishing cells of the normal erythrocytic series chiefly on the basis of cytoplasmic maturation, though simple and generally accepted, is replaced by a proposed differentiation which would be most difficult to follow. Thus, an attempt at orderliness is likely to bring confusion instead.

It is difficult to escape the conclusion that the recommended terminology would only add to the terms that the student must learn and would increase verbiage where there is sufficient already. No one will be better off in reading literature

\* For another editorial on *Names for Blood Cells* see *Lancet* 1: 486 (March 19) 1949

published hitherto and there will still be debate as to whether a given cell is one thing or another

Differences in terminology have arisen mainly because of differences in interpretation of observations made under a variety of conditions. It is reasonable to expect that as new knowledge is gained, agreement will come naturally as there is better understanding. Terms may then be modified by the normal process of evolutionary selection rather than through arbitrary definition. Emphasis, in short, should be placed on advancing knowledge rather than in too much concern about names.

Since the new terminology is not readily and wholly acceptable, nothing can be gained by its introduction at this time. Haste will but make more difficult the acceptance of terms which time and repeated discussions of all those concerned, including interested individuals in all English-speaking countries, might make possible in the future.

MAXWELL M. WINTROBE, M.D.

#### REFERENCES

<sup>1</sup> Am J Clin Path 18:443 1948

<sup>2</sup> Am J Clin Path 19:56 1949

<sup>3</sup> Blood 4:89 1949

#### LETTERS TO THE EDITOR

##### TO THE EDITOR

In the January issue of *Blood* a condensation of the first two Reports of the Committee for Clarification of the Nomenclature of Cells and Diseases of the Blood and Blood Forming organs was published. Since I am not convinced of the soundness with which this unilateral approach has been conducted, I would like to bring certain things to your attention.

Hematology is international in its scope and as a consequence its terminology is not the property of any one country. As in other scientific disciplines, the ultimate goal of uniformity in nomenclature is certainly one which is desired by all. It is for this very reason that the constitution of the International Society of Hematology lists one of its purposes— to attempt to standardize on an international scale hematologic methods and nomenclature.

Such an undertaking would undoubtedly enlist not only the services of clinical pathologists, but also those of embryologists, histologists, physiologists, zoologists, tissue culturists, immunohematologists, and others whose work might be influenced by an alteration in hematologic terminology. The approach must be multilateral from the start. Groups serving on this committee should be provided with extremely accurate illustrations of cells under discussion. Furthermore, these illustrations should include the range of variability of cell types.

This is not the first attempt by Americans to alter terminology. As a matter of fact, some of the confusion which is disturbing at the present time is the primary fault of American hematologists. In 1915 Doan, Cunningham and Sabio with very good intentions wrote. The terminology in hematological literature has become so confused, different investigators using the same designation for wholly different histological entities or the same histological entity being designated by a variety of terms, that it becomes necessary to define the limited sense in which certain names already in the literature will be used in this paper. Then in the case of erythropoiesis, they disregarded this by using the term megalo-blast in a manner quite different from its accepted usage by such leading hematologists of that period as Downey, Ferrata, Maximow, and Naegele. Additional confusion in American hematology was created by Peabody's unequivocal acceptance of Doan, Cunningham and Sabio's theory and by Isaacs and Osgood propounding theories of a similar nature. It is unfortunate that Doan, Cunningham and Sabio advanced

their theory for avian and mammalian erythropoiesis during a period when so much emphasis was being placed on the pathologic physiology of blood forming organs. If one takes time to read British, French, German, Swiss, Italian, Scandinavian and Latin American contemporary hematologic literature, it soon becomes apparent that Doan et al., Isaacs and Osgood have placed certain phases of American hematology in a very bad light.

For some time Europeans, Latin Americans and some Americans have recognized the inadequacy of the theory for red cell genealogy as proposed by Doan et al., Isaacs and Osgood. Apparently Osgood too recognizes that something is amiss and believes it can be rectified by avoiding certain terms like the megaloblast, for example. The importance of this cell type is more clearly understood in Europe today than it has ever been before. In order to illustrate this importance, consider the following from an unpublished manuscript:

"The megaloblast problem has many ramifications which affect, in varying degrees, the thought in quite a few branches of medical science. Zoologists who do research in comparative hematology have pointed out that megaloblasts and the first circulating mammalian embryonic red blood cells should be called ichthyoid, since they resemble the permanent red cells of fish and amphibians. The embryologist is interested in whether or not these cells are present only in the yolk sac during the prehepatic period of embryogenesis, or whether they are also found in the embryonic liver, spleen and bone marrow. They would also like to know whether the embryonic and pathologic red blood cells are identical. Some histologists teach that megaloblasts, derived from endothelium of the adult, are present in normal bone marrow and act as normal precursors for definitive erythrocytes, whereas other histologists consider that they belong quite definitely to the realm of pathology. In the latter, many general clinical pathologists maintain that the presence of megaloblasts are pathognomonic for all liver principle deficiency anemias, while, on the other hand, some do not consider their presence unusual in any type of anemia. Experimental pathologists and internists have been attempting to produce in laboratory animals a condition which would simulate pernicious anemia of humans and some have purported to have produced a megaloblastic bone marrow. Some physiologists consider that these cells function as the first hemoglobin synthesizing units under normal conditions and the biochemists are confronted with the problem of determining whether or not hemoglobin is identical under all conditions. Pharmacologists, who are interested in the bioassay of antipernicious anemia preparations, would like to know whether or not megaloblasts are the only red cells which will respond in the presence of these substances. Furthermore, they are also concerned with determining what portions of specific molecules of these substances will cause megaloblasts to disappear from the marrow of pernicious anemia patients. They also ponder the question of why all patients with a megaloblastic marrow do not respond to the same specific therapy. Some clinicians are interested in the megaloblast problem because the presence of such cells in a patient's marrow indicates to them a need for the administration of specific therapy which, in most cases, must be maintained at an optimal level throughout the remainder of the patient's life. And, needless to say, the absence of these cells from the sternal marrow of a patient with severe anemia affords the rationale for an entirely different therapy. Lastly, the hematologist has at least a theoretical interest in many, if not all, of these phases pertaining to the megaloblast problem.

To some it may seem that all of this is so much hogwash and that the difficulty might be solved readily by a change in terminology. Therefore, let us cast aside all hematologic terminology and designate the earliest cell recognizable as a hemoglobin synthesizing unit as red cell No. 1. The next step would be to study this cell under embryonic, fetal, normal adult, pathologic and experimental conditions, determining the morphologic features of its nuclear pattern and cytoplasm. If red cell No. 1 has the same attributes under all of these conditions, then we are justified in selecting the most appropriate term as a label for all. On the other hand, if red cell No. 1 differs under embryonic, normal adult and certain pathologic conditions, we would not be justified in grouping all of these cells together. In addition to this, if there are constant morphologic differences, then cytochemical, physiologic and biologic studies should be made to determine the underlying basis for them. Since there are morphologic and other differences between some of these cells, they should be called red cell No. 1 under embryonic conditions, red cell No. 1 under normal adult conditions, and red cell No. 1 in liver principle deficiency anemias during relapse. In the latter instance, it has been appropriate to refer to these cells as megaloblasts because that term—good or bad—was first applied to them by Ehrlich in 1880.

Just for the sake of argument, let us assume that the proposed terminology has been accepted by all. Will it change the megaloblast normoblast problem? No, it will not! In its place some American clinical hematologists and pathologists will continue to recognize morphologic differences between a pernicious anemia type prorubricyte and a rubriblast and others will not. The problem will remain just so long as some American hematologists either fail to recognize minute but constant differences in nuclear pattern or fail to interpret them properly.

The proposed terminology for red cells might have done better by utilizing good Anglo-Saxon terms like large, medium and small. The proposed terms of rubriblast and prorubricyte are regrettable in that they are hybrid. A Latin-Greek red cell gives rise to a Greek red cell. However, it is consoling to know that megalocytes are not to be avoided and that it is possible for them to come from metarubricytes. The suggested terminology will result in one more complex than existing ones, for example, megaloblasts become pernicious anemia type prorubricytes. In anatomic nomenclature there is a tendency to avoid cumbersome terms and not create them. Why should, for example, the pectineal part of the inguinal ligament be used when lacunar ligament is available?

In conclusion, it is generally recognized that problems of nomenclature or classification become less complex when more is learned about the various attributes of the subject in question. Anatomists have more than a casual interest in hematologic nomenclature because they are responsible for teaching embryology and histology to medical students. It would be very unfortunate to teach two terminologies—one for preclinical and the other for clinical courses.

Oliver P. Jones  
Professor of Anatomy,  
The University of Buffalo, School of Medicine

Dr Wintrobe's Editorial and Dr Jones' letter were referred to Dr E. E. Osgood, who replied as follows:

#### TO THE EDITOR

Both Doctor Wintrobe and Doctor Jones admit a state of confusion in definitions and terminology has existed in the field of hematology. Their criticisms, which are not clearly answered in the reports of the Committee, condense to the following four statements:

1. *Next year we will know more, therefore we should wait.*

*Answer:* This has been and will always be true. If we were to wait until all is known or until 100 per cent agreement is reached, nothing would ever be done.

2. *They and some others criticize the term progranulocyte, the terms selected for the erythrocytic series and the qualifying adjective phrase, pernicious anemia type.*

*Answer:* It is admitted that promyelocyte would be more consistent with other terms selected for cells of the granulocytic series. However, the Committee recommended the term, progranulocyte, because the definition accompanying it excludes cells containing neutrophilic, eosinophilic or basophilic granules. The term promyelocyte has been used for cells variously defined as containing 10 per cent, 30 per cent or 50 per cent of their full quota of such granules, and these are lines of division between stages of differentiation which two observers cannot exactly duplicate.

The definitions and terms in current use for the erythrocytic series were fully discussed at each of three meetings and the recommended terms resulting were agreed on as being the best solution to an admittedly difficult problem. The question is not, "Is this solution ideal?", but is rather, "Can anyone suggest a better solution?" General agreement on *one* definition and *one* term for each cell stage is more important than the particular term selected. These were the terms and definitions recommended. Agreement is more easily reached around a conference table, so it seems most unfortunate that neither Dr. Jones nor Dr. Wintrobe found it possible to attend any of the Committee meetings to which they were invited. Dr. Hal Downey, whom Doctor Jones mentions in his letter, was present at the meetings and fully concurs in the recommendations. The 30 members of the Committee who have approved the recommendations of the second report include competent men in most of the fields mentioned in Doctor Jones' letter. During some of the meetings, atlases of hematology as well as blood and marrow smears

and microscopes were available to all discussants so that morphologic differences and similarities could be visually evaluated during the process of reaching agreement for recommended terms and definitions

Both letters imply that use of these terms binds one to a particular theory. One of the most fundamental principles guiding Committee decisions and emphasized in the reports has been to avoid any attempt to settle around a conference table anything which could be settled only by investigation. If anyone wishes to teach his students that a polychromatic erythrocyte is less differentiated than a normochromatic rubricyte he can express that opinion clearly in this recommended terminology.

One of the major weaknesses of other terminologies has been that they failed to distinguish between stages of differentiation and the disappearance of ribonucleoprotein with simultaneous appearance of hemoglobin in the cytoplasm. With the recommended terms both can be clearly indicated. Dr Wintrobe pleads for more consistency in the terms for the granulocytic series but would retain the suffix *-blast* in the erythrocytic series for a cell with a pyknotic partially extruded or partially autolyzed nucleus which does not fit the criteria for any other *blast* stage. One needs to ask but one question regarding the term pernicious anemia type versus megaloblastic. Even if megaloblastic had only one definition would it not be clearer to the student of medicine studying it for the first time to learn about pernicious anemia type granulocytes and pernicious anemia type marrow picture than about a megaloblastic marrow picture? Certainly one could not speak of megaloblastic granulocytes yet the morphologic changes are just as striking as those in the erythrocytic series.

### 3 *A special atlas is necessary*

*Answer.* If the definitions are carefully read—and these definitions are just as important as the terms—it will be seen that the criteria for differentiation of the stages are clearly illustrated in every atlas of hematology that has ever been published. The Committee clearly recognizes that all subdivision is arbitrary and that an infinite number of subdivisions would be possible. They selected that number of subdivisions which in their experience was clinically and diagnostically useful and tried to phrase definitions that would put the same cell in the same category when seen by different observers, but made provision for as much further subdivision through the use of modifying adjectives as might be needed for any investigative purpose.

### 4 *The recommendations should be international before they are published*

*Answer.* The problem seems sufficiently difficult to settle in one language at a time. It is the sincere hope of the Committee that other language groups will form similar committees and that they will give serious consideration to the advisability of selecting the same definitions at least and to achieving a comparable nomenclature.

The other points raised in the two letters are clearly answered in the text matter of the reports of the Committee. The Committee reports were circulated before publication to all Committee members whether or not they were in actual attendance at meetings. These published reports<sup>1, 2, 3</sup> represent the combined efforts of a number of persons with the approval of the majority of the members of the Committee; they are not the recommendations of any one individual.

The terms to be avoided are not synonymous in most instances with the term to be used. They are merely terms that have been used by some for the cells included under the terms recommended and defined in the reports. The Committee reports are *recommendations* only and provision has been made to review and revise terminology periodically. It is not to be expected that they will receive 100 per cent acceptance in areas where scientific freedom exists. A statistical analysis of the response to the recommended nomenclature which has been received by Committee members and the American Society of Clinical Pathologists has not yet been made, but it will be brought into presentable form and the result will be made public in a future Committee report.

In conclusion it is felt that to debate the values of any nomenclature in the scientific press can result only in the amassing of a large body of print and a loss of considerable time. The interested reader of these letters should be referred to the published reports of the Committee for therein are included all of the purposes and guiding principles. If the recommended nomenclature has merit it will be used if it lacks merit it will atrophy from disuse. The present indications—obtained from verbal comments and letters—are that the recommended terms and definitions are being widely adopted.

As stated in the Committee reports the primary purpose of this Committee has at all times been to clarify hematologic terms for the benefit of the medical profession as a whole and future students of medi-

cine and related sciences, rather than for the relatively small proportion of the present medical profession which devotes most of its time to hematology

For the Committee for Clarification of the  
Nomenclature of Cells and Diseases of  
the Blood and Blood Forming Organs  
EDWIN E. OSGOOD, M.D. Chairman  
ROY R. KRACKE, M.D. Dean The  
Medical College of Alabama  
F. J. HECK, M.D. Mayo Clinic

#### REFERENCES

- <sup>1</sup> First report of the Committee for Clarification of the Nomenclature of Cells and Diseases of the Blood and Blood Forming Organs *Am J Clin Path* 18 443-450 1948
- <sup>2</sup> Second report of the Committee for Clarification of the Nomenclature of Cells and Diseases of the Blood and Blood Forming Organs *Am J Clin Path* 19 56-60 1949
- <sup>3</sup> Condensation of the first two reports of the Committee for Clarification of the Nomenclature of Cells and Diseases of the Blood and Blood Forming Organs *Blood* 4 89-96 1949
- <sup>4</sup> Recommended terms and definitions for cells of the leukocytic erythrocytic and thrombocytic series *J. A. M. A.* 139 175-176 January 15 1949

A subsequent letter received from Dr. Jones indicates that he was unable to attend two of the last meetings, and that he does not agree on all points with the report. The following letter on the subject was received from Dr. Frank H. Bethell:

#### TO THE EDITOR:

The statements of the chairman of the Committee on Classification of Nomenclature of Cells and Diseases of the Blood and Blood Forming Organs made in answer to the criticisms of Doctors Jones and Wintrobe have my whole-hearted endorsement. I believe that the publication of these letters will serve a useful purpose if it leads to a broader understanding of the objectives and achievements of the Committee. As Dr. Wintrobe says, the opposition to the recommendations of the Committee has been expressed for the most part in informal conversations. My participation in some of these discussions has convinced me that the discussants, with few exceptions, have not been well informed on the content of the Committee's reports. I should like to urge that every interested person, before he takes a position in this controversy, read carefully the published reports of the Committee with particular attention to the definitions.

FRANK H. BETHELL, M.D.  
University of Michigan, Thomas Henry Simpson  
Memorial Institute for Medical Research

#### FURTHER COMMENT ON NOMENCLATURE DISCUSSION

From the vantage point of the Editorial chair, there seems to be a good deal of merit in both points of view regarding the proposed revision and systematization of hematologic nomenclature. Although faint echoes are heard in this discussion of the frequently polemic articles which were seen in *Folia Haematologica* years ago, it can be stated that the proposed system of nomenclature, as worked out by a serious group of well-intentioned observers, is not only in the interests of simplicity, but slanted frankly for the students and the younger generation of physicians. Although some of us may dislike to have terms changed or systematized, many of



the younger men in the field have evidently taken to the newer terms without too much difficulty, even to the seemingly outlandish ones of rubricytes and the like. Certainly, consistency is always something to be applauded so why not use for leukemia the terms myelocytic, lymphocytic, and monocytic rather than *myelogenous*, *lymphatic* and *monocytic*? It is admittedly easy to slip into this particular consistency but on the other hand, one finds it hard to take to one's bosom the rubriblast or to understand the actual need for its use. Therefore, it is good to note Dr. Osgood's statement that the proposed system of nomenclature is by no means rigid and that by a process of selection the fundamentally correct and the simple terms will be retained and the wrong and the difficult ones will atrophy from disuse. The members of the Committee are to be congratulated for the vast amount of time and patience they have spent around the conference table. They are doubtless correct in having obtained the impression that some of their critics might have been less critical had they spent some time with them in discussing their problems. In any event, there can be little doubt that out of all this great effort, at least some good will ensue to the innocent bystander in hematologic nomenclature.

WILLIAM DAMESHEK, M.D.

# ABSTRACTS

JOSEPH F ROSS M D , *Editor*

ABSTRACTERS

HELEN W BELDING, M D , Winston Salem N C  
SHEILA T CALLENDER M D Oxford England  
GEORGE E. CARTWRIGHT M D Salt Lake City  
ROGER C. CRAFTS Ph D , Boston  
CHARLES P EMERSON M.D Boston  
SOLOMON ESTREN M.D New York  
ROBERT S EVANS M D San Francisco

CLEMENT A FINCH M D , Boston  
OLIVER P JONES Ph D , Buffalo  
CONRAD MATER M D Zurich, Switzerland  
MILOS NETOUSEK, M D Prague Czechoslovakia  
JEAN P SOULIER, M D , Paris  
RAMON M SAREZ, M D , San Juan Puerto Rico  
JAN WALDENSTRÖM M D Upsala Sweden

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## BLOOD COAGULATION

STABILITY OF PROTHROMBIN AND AC-GLOBULIN IN STORED HUMAN PLASMA AS INFLUENCED BY CONDITION OF STORAGE J L Faley A G Watt and W H Stegers From the Department of Physiology Wayne University College of Medicine Detroit Michigan Am J Physiol 154 122-133, 1948

Normal plasma has been shown to contain, among other things a globulin factor which affects the transformation of prothrombin to thrombin—a plasma accelerator substance called Ac globulin. The present report concerns the stability of this substance and of prothrombin itself under various conditions. Under specific conditions human venous blood was drawn into citrate or oxalate anti-coagulant mixtures and then after a variety of manipulations (centrifugation at various speeds storage for various periods of time use of various concentrations of oxalate or citrate) tested for the amounts of contained prothrombin and Ac globulin.

For testing purposes Ac globulin was determined by a previously described method in which prothrombin, thromboplastin and calcium are present in controlled amounts, so that the rate of thrombin formation measures the amount of Ac globulin. The amount of prothrombin was determined (1) by a standard two-stage method in which saline appears as a diluent and (2) by a modified two-stage method in which saline is replaced by bovine serum (containing of course Ac globulin).

In both oxalated and citrated plasma the prothrombin activity did not change for a period of several days. After this period, the original two-stage method detected a progressive fall in prothrombin. However by the modified method—in which Ac globulin was added—the amount of prothrombin was still unchanged for as long as fifty-six days. It seemed obvious therefore that the apparent fall in prothrombin was illusory and that the fall was due to a loss of Ac globulin and not prothrombin itself from the stored plasma. In addition the apparent (illusory) prothrombin fall occurred earlier and was more marked in oxalated than in citrated plasmas i.e., Ac globulin was less stable in oxalated than citrated blood.

In a further experiment a sample of oxalated plasma which originally had a prothrombin content of 290 units/cc was found after 53 days of storage to have an apparent prothrombin content by the original two-stage method of 19 units/cc. When this determination was modified by the addition of (a) bovine serum or (b) pure Ac globulin, or (c) an extract of bovine platelets then the prothrombin content was found still to be 290 units/cc. It thus seems that not only serum but also platelets, contain a prothrombin accelerating substance. However platelets were also found to contain a factor which decreased the stability of Ac globulin, this factor was apparently operative after the platelet Ac globulin supply had been dissipated (24 hours of storage).

These and further similar experiments will compel revision of the simple schemata of blood coagulation. It seems likely however that the plasma, serum, and platelet Ac globulin substances (which are similar) are the same as Quick's prothrombin A and as Owren's factor V and that they play an important role during the initial stages of coagulation.

S.E.

CONCENTRATION OF PROTHROMBIN AND AC-GLOBULIN IN VARIOUS SPECIES *R C Murphy and W H Siegers* From the Department of Physiology, Wayne University College of Medicine, Detroit Michigan. *Am J Physiol* 154 134-139 1948

The authors determined the concentrations of prothrombin and Ac globulin in the bloods of various animals, including man and noted that there was a wide variation from species to species. For example, it was found that although the plasma prothrombin content in dogs man and guinea pigs was the same (200 to 300 units/cc) the plasma Ac globulin of the dog measured 150 units/cc that of man 12 units/cc and that of the guinea pig 30 units/cc. Since the Quick one-stage method of prothrombin estimation measures not the amount of prothrombin but the amount of prothrombin plus the rapidity of its conversion to thrombin (this conversion depends to a large degree upon Ac globulin) it can be seen that the differences in various species between the one stage and the two-stage methods of prothrombin determination may well be due to the marked variations in concentration of Ac globulin in various species. Thus dog, man and guinea pig have identical prothrombin contents (two-stage method) but widely divergent prothrombin times (one stage method) due probably to the different Ac globulin concentrations found.

Man has a low plasma Ac globulin activity, and therefore a relatively high ratio of prothrombin to Ac globulin. Hence the authors suggest, there may be a relatively narrow margin of safety beyond which hypoprothrombinemia may occur.

S.E.

PLATELET EXTRACTS, FIBRIN FORMATION AND INTERACTION OF PURIFIED PROTHROMBIN AND THROMBOPLASTIN *A G Ware J L Fabry and W H Siegers* From the Department of Physiology Wayne University College of Medicine Detroit Michigan. *Am J Physiol* 154 140-147 1948

Analysis of extracts of bovine platelets led to a revision of the relatively naive concept that platelets initiate blood coagulation by the liberation of initial amounts of thromboplastin. Actually a small amount of thromboplastin was found in platelets, but it was only a very small amount. In addition two other substances were present: (1) an Ac globulin type of substance and (2) a new factor called platelet factor 2.

1. Platelet extract was found to contain something which accelerated the conversion of prothrombin to thrombin in the presence of thromboplastin and calcium. This substance acted similarly to serum Ac globulin and was quantitatively identical with serum Ac globulin in its ability to convert prothrombin into thrombin. In addition platelet Ac globulin and serum Ac globulin were both similarly precipitated by half saturated ammonium sulfate and were both destroyed by heating to 53 C. On the other hand there was a difference between the two substances in their length of stability at 53 C. and more dramatic, only platelet Ac globulin could be sedimented in the ultracentrifuge. Hence it was concluded that platelet Ac globulin and serum Ac globulin are probably two different proteins with similar prothrombin activating activities. The authors estimated that some 5 per cent of the total accelerator activity comes from the platelets.

2. Platelet extracts were found to contain a previously undescribed action or substance which hastened the action of thrombin on fibrinogen. The factor was inferred from the following type of data:

thrombin + fibrinogen = clot in 16 seconds
platelet extract + thrombin + fibrinogen = clot in 11 seconds
platelet extract + fibrinogen = no clot in 10 minutes

This substance was rapidly diluted out. It is still under investigation.

It is pointed out that neither serum Ac globulin nor platelet Ac globulin is absolutely necessary for the production of thrombin; these substances apparently act merely as catalysts. A detailed schema is presented in which the roles of these accelerator substances in the initial stages of coagulation is incorporated. The schema of course is still speculative.

S.E.

PROTHROMBIN CONVERSION FACTOR OF DICUMAROL PLASMA. *C A Owen and J L Bollman* From the Division of Experimental Medicine, Mayo Foundation, Rochester Minnesota. *Proc. Soc. Exper Biol & Med* 67 231-234, 1948

Data obtained from experiments on dicumarolized dogs suggests that the hemorrhagic diathesis produced by dicumarol is attributable not alone to a disappearance of prothrombin but also to the loss of a factor, the function of which is to facilitate the conversion of prothrombin to thrombin. Variations in the concentration of this conversion factor present in plasma serum or serum pseudo-globulin may explain the familiar discrepancies in the results of one and two-stage methods of estimating prothrombin activity. It may also account for the therapeutic efficacy of serum in the treatment of cattle with sweet clover disease, a phenomenon otherwise difficult to explain.

C. P. E.

ACTION DE LA PHENYL-INDANE DIONE SUR LE TAUX DE LA PROTHROMBINE I. ETUDE EXPERIMENTALE SUR LE LAPIN J. P. Soulier and J. Guiguen II. UTILISATION EN CLINIQUE HUMAINE (EFFECT OF PHENYL-INDANE DIONE ON PROTHROMBIN LEVELS I. EXPERIMENTAL STUDIES ON THE RABBIT II. USE ON HUMANS.) J. Guiguen and J. P. Soulier *Rev. Hemat.* 3: 180-195, 1948.

In the first series of experiments using 16 rabbits, the authors found that phenyl indane-dione (P.I.D.) had a very marked effect on prothrombin level. Doses of 10 to 20 milligrams per kilo produced a decrease of prothrombin to a level of 30 to 40 per cent, this effect being reached before the eighteenth hour. There was no modification of platelets clot retraction or fibrinogen level. Higher dosage did not produce greater hypoprothrombinemia and the authors did not find any hemorrhages even with a dosage ten times the standard dosage. The lethal dose was well over 600 mg./kilo which gave a very high safety margin. Histologic examinations of the rabbits given very high doses of P.I.D. (under 400 mg./kilo) did not show histologic injuries.

The P.I.D. was used in the prevention of thrombosis in 43 women after pregnancy. In all these cases doses of 10 to 20 mg./kilo yielded a very constant decrease of prothrombin level. The decrease began earlier than with dicumarol about the twelfth hour and the full effect was obtained between the twenty-fourth and the forty-eighth hour which is a 30 to 40 per cent level. Return to a normal level was quite constant and 100 per cent prothrombin was reached by about the ninety-sixth hour.

This constancy in the chronology is very different from that observed with dicumarol. Individual susceptibility to the drug seems also to be less important than in the case of dicumarol.

In 2 cases, the P.I.D. was given to patients with known thrombophlebitis (every 3 days 10 mg./kilo). This dose was effective in controlling the prothrombin level around 30 per cent. The patients' state was in both cases favorably affected. In the 41 cases where the drug was given prophylactically, no phlebitis was observed.

In contrast with these advantages the complete inactivity of vitamin K<sub>2</sub> even in huge doses and even when given prior to the administration of the P.I.D. must be emphasized. But this fact is perhaps of minor importance since in no case was hemorrhage or hypoprothrombinemia of less than 10 per cent observed.

J. P. S.

THE RELATIONSHIP OF HEPARIN ACTIVITY TO PLATELET CONCENTRATION C. L. Conley, R. C. Hartmann and J. S. Lelley. From the Department of Medicine, Johns Hopkins University and Hospital, Baltimore. *Maryland Proc. Soc. Exper. Biol. & Med.* 69: 284-287, 1948.

To evaluate the significance of the reported increased susceptibility of thrombocytopenic blood to heparin (*Ann. Int. Med.* 27: 382, 1947) these investigators studied the clotting times of platelet free and platelet-rich plasmas prepared in silicone coated apparatus which were mixed in different proportions and contained graded concentrations of added heparin. The data presented justifies the conclusion that the magnitude of the clot inhibitory effect of heparin is inversely proportional to the number of platelets present and that the increased susceptibility of thrombocytopenic purpura blood to the action of heparin is attributable to the reduced platelet concentration rather than to a supplemental anticoagulant effect introduced by the presence of hypothetical heparin like substance. The results further suggest that the concentration of active heparin normally present in plasma is minute i.e. 0.0005 mg./ml. or less.

C. P. E.

CONCERNING THE RELATION BETWEEN PITUITARY ADRENOCORTICOTROPHIN AND THE CIRCULATING BLOOD PLATELETS M. A. Greer and B. R. Brown. From the Joseph H. Pratt Diagnostic Hospital and the Depart-

ment of Medicine Tufts Medical School, Boston Massachusetts Proc Soc. Exper Biol & Med 69 361-362, 1948

Since an increase in platelet concentration is usually found in the peripheral blood attending infection, trauma hemorrhage, and asphyxia, conditions which stimulate a release of pituitary adrenocorticotrophin (ACTH), the possible effect of the latter was tested in rats injected either with hog pituitary tissue or purified ACTH. A preparation of the latter was also given by repeated intramuscular injection to five human subjects including three normal individuals one male and two female, a young woman with hypopituitarism and another with thrombocytopenic purpura following an unsuccessful splenectomy. No change in platelet count was detected following these procedures. Moreover although pituitary ACTH is capable of causing a dissolution of lymphoid tissue with peripheral lymphopenia (Endocrinology 35 1, 1944), no reduction of circulating lymphocytes occurred in these experiments the only hematologic effect noted being a transient polymorphonuclear leukocytosis.

C.P.E.

THE CHEMICAL STATE OF THE CALCIUM REACTING IN THE COAGULATION OF BLOOD A. J. Quick and M. Stefanski From the Department of Biochemistry, Marquette University School of Medicine, Milwaukee, Wisconsin J Gen Physiol 32 191-202, 1948

For many years it has been generally accepted that ionized calcium is essential for coagulation. The use of amberlite which quantitatively removes calcium from the blood and other new techniques have been utilized by the authors to reinvestigate some of these problems. Sodium oxalate and citrate act in different manners. The oxalate not only precipitates ionized calcium but it also removes it from a compound which is essential for coagulation. Citrate combines with prothrombin and renders it inactive. The addition of magnesium or strontium restores the prothrombin to its original state. Studies of prothrombin activity under various types of condition have suggested the presence of a labile factor which is indispensable for coagulation and unstable in decalcified plasma.

O.P.J.

EFFECT OF AMINOPHYLLIN ON THE COAGULATION OF HUMAN BLOOD D. W. Blood and M. C. Patterson From the Department of Medicine Columbia University College of Physicians and Surgeons and the Medical Service of the Presbyterian Hospital, New York City, New York. Proc Soc. Exper Biol & Med 69 130-133, 1948

Experiments are reported which fail to confirm published reports ascribing to aminophyllin (theophyllin-ethylenediamine) a thromboplastic action with an accelerating effect on blood clotting which might predispose to intravascular thrombosis. Following the administration of aminophyllin orally or by vein no statistically significant changes were detectable in the clotting time or prothrombin activity of hospital patients with normal hepatic function and hematological findings.

C.P.E.

PROLONGATION OF ACTION OF HEPARIN J. J. Verzeletti, L. N. Sussman and M. J. Marder From the Medical Service Beth Israel Hospital, New York City, New York. J. A. M. A. 138 747-748 1948

In search for a form of heparin which might have a more prolonged action in the body than those currently available the authors devised a concentrated form of heparin (200 to 300 mg per cc of aqueous solution) emulsified in a mixture of cholesterol derivatives paraffin oil and beeswax. The use in this form of about 2 milligrams of heparin per pound of body weight resulted in satisfactory prolongation of the coagulation time for seventeen to twenty-four hours after a single injection. There were no toxic effects and no hemorrhagic phenomena with high coagulation times. According to the authors pain was negligible. Compared with this method the use of concentrated aqueous heparin increased the coagulation time for only six hours and the use of heparin dissolved in Pitkin's menstruum was associated with severe pain at the site of injection.

Although the authors list expensiveness as a disadvantage in the use of other forms of heparin they do not mention the relative cost of the current preparation.

S.E.

**HEREDITARY HEMORRHAGIC TELANGIOECTASES ASSOCIATED WITH PULMONARY ARTERIOVENOUS FISTULA IN TWO MEMBERS OF A FAMILY** *J H Meyer and A J Ackerman* From the Brooke General Hospital, Brooke Army Medical Center Fort Sam Houston, Texas *Ann Int Med* 29 775-802, 1948

This is a well documented article (57 references) in which the literature on hemorrhagic hereditary telangiectasia is reviewed. A family with the disease is described and particular reference made to the pulmonary arteriovenous fistula which occurred in two cases. Clinical, pathologic, physiologic and roentgenologic aspects of this complication are discussed.

C A F

**HEMORRHAGE** *G Schwartzman and others* From various centers *Ann New York Acad Sc* 49 483-660 1948

This monograph includes, among other topics, the following discussions on hemorrhagic disorders: mechanism of coagulation, heparin, vitamin K and other vitamins, pseudohemophilia, hypoprothrombinemia and the metabolic alterations following hemorrhage.

S.E.

## ERYTHROCYTE MORPHOLOGY AND PHYSIOLOGY

**THE STRUCTURE OF UNSTAINED RETICULOCYTES** *G Brecher* From the Pathology Laboratory, Experimental Biology and Medicine Institute, National Institute of Health, U S Public Health Service, Bethesda, Maryland *Proc Soc Exper Biol & Med* 69 89-90 1948

By means of phase microscopy, according to the author, it is possible to demonstrate the presence of intracellular granules and rods exhibiting Brownian movement, in unstained wet preparations of mouse blood diluted with hypotonic oxalate solutions. Similar findings were obtained on examining dried unfixed smears mounted in 10 per cent formalin or 1.2 per cent ammonium oxalate. The number of cells presenting these characteristics corresponded with the number of reticulocytes counted by routine methods. Moreover, their identification as reticulocytes was confirmed by the device of adding brilliant cresyl blue or new methylene blue to the oxalate diluent; these rods and granules being incorporated into stainable reticulum. Similar observations have been made in preliminary studies of human blood.

C P E.

**SIDEROCYTE COUNTS IN THE BLOOD OF NORMAL AND PREMATURE INFANTS** *H P Wright and D G Edmonds* From the Obstetric Unit, University College Hospital, London, England *J Path & Bact* 60 342-344 1948

Fifty normal full term infants and 7 premature infants were examined daily for the first 8 days of life. Parallel observations were made using the 2a' dipyrindyl staining technique and the prussian blue method. Reticulocytes were also counted. The percentage of granule containing cells (siderocytes) was very similar with both methods. The number in the blood of normal newborn infants gradually decreased during the first 8 days to approximate the value reported for normal adults. There was a rise of siderocytes from the second to fourth days in infants exhibiting physiologic jaundice. Premature infants had an even higher siderocyte percentage with the maximum occurring on the fourth day.

O P J

**VISCOSITY STUDIES OF ERYTHROCYTES FROM PERSONS WITH SICKLE CELL DISEASE** *W M. McCord, W H Kelley, P K Switzer and F B Culp* From the Departments of Chemistry and of Medicine, Medical College of the State of South Carolina and of Roper Hospital, Charleston, South Carolina *Proc Soc Exper Biol & Med* 69 19-22, 1948

The increase in blood viscosity attending the sickling of red cells previously demonstrated in cases of sickle cell disease (*J Clin Investigation* 19 788 1940) has been utilized by these authors as the basis for a viscosimetric method adaptable for the study of this trait. Employing an Ostwald viscosimeter and testing preparations of red cells washed and suspended in Tyrode's solution, an increased viscosity was observed following equilibration of the samples with nitrogen or carbon dioxide, an effect which was prevented by preliminary equilibration with carbon monoxide but not effected by alterations of pH, by

repeated washing of the erythrocytes with saline solution or by the addition of heparin oxalate or cyanide

C.P.E.

THE QUANTITATIVE DESCRIPTION OF THE FRAGILITY OF THE ERYTHROCYTE AND ITS APPLICATION TO THE STUDY OF ACHOLURIC JAUNDICE *G. Discombe* From the Department of Pathology St Bartholomew's Hospital London England *J. Path. & Bact.* 60 315-322 1948

Red cell fragility may be quantitated by counting the number of erythrocytes which survive treatment in a given solution or by measuring the hemoglobin liberated. For precise work the hemoglobinometric method using a photo-electric absorpiometer is essential. That concentration of salt which produces 50 per cent lysis is designated the mean corpuscular fragility or M.C.F. When plotted on arithmetic probability paper the points for normal subjects fall on or close to a straight line. Fragility curves from patients with classical acholuric jaundice vary considerably in form and suggest two or three groups of cells differing in M.C.F. and its standard deviation. The affected members of one family suffering from acholuric jaundice yielded straight line curves. It is suggested that this family has a genetically distinct form of the disease.

O.P.J.

EFFECT OF BAL ON COBALT INDUCED POLYCYTHEMIA IN RATS *L. O. Jacobson, E. K. Marks and E. Gatten* From the Argonne National Laboratory and the Department of Medicine University of Chicago Chicago Illinois *Proc. Soc. Exper. Biol. & Med.* 69 84-86 1948

In view of the demonstration (Fed. Proc. 5 126 1946; *J. Biol. Chem.* 165 723 1946; *Cancer Res.* 4 497 1946) that the toxic properties of cobalt are related to its effect on —SH groups, the action of BAL, a protector of —SH groups in arsenical poisoning, was studied with reference to the possible prevention of cobalt induced polycythemia in rats. No such inhibitory influence of BAL on the development of polycythemia was demonstrable when the drug was supplied three times weekly in a dose of 0.2 mm/kg concomitantly with cobaltous chloride.

C.P.E.

OXYGEN SATURATION OF STERNAL MARROW BLOOD WITH SPECIAL REFERENCE TO PATHOGENESIS OF POLYCYTHEMIA VERA *L. Berk, J. H. Burchenal, T. Wood and W. B. Castle* From the Thorndike Memorial Laboratory Second and Fourth Medical Services (Harvard) Boston City Hospital and the Department of Medicine Harvard Medical School Boston Massachusetts *Proc. Soc. Exper. Biol. & Med.* 69 316-320 1948

The authors performed gasometric measurements of the oxygen content and capacity of blood samples obtained from the sternal marrow cavity of 23 patients with polycythemia vera, 6 with secondary polycythemia, 12 with chronic anemia, and 11 with leukemia and myeloid metaplasia. Control studies were conducted on 34 individuals whose hematologic status was essentially normal. The range of percentage oxygen saturation was similar in all groups studied with the exception of patients with secondary polycythemia in whom there was a reduction of marrow oxygen saturation corresponding to the relative unsaturation of the peripheral arterial blood. Although data were obtained in some cases suggesting an increased oxygen utilization relative to the blood flow, the technique of sampling used by the authors precluded in their opinion a satisfactory demonstration of local hypoxia, to which increased red cell production in anemic states has been attributed and which may be responsible for chronic erythropoietic stimulation in patients with polycythemia vera.

C.P.E.

SOME ASPECTS OF RED CELL PRODUCTION AND DESTRUCTION *Editor R. W. Munst, E. Ponder and others* From the American Museum of Natural History New York. *Ann. New York Acad. Sc.* 48 577-704 1947

This excellent monograph comprises six articles on the architecture, production and destruction of the red cell. There are four comprehensive but concise reports on the fundamental aspects of red cell cytochemistry, endocrinology and erythropoiesis, experimental hemorrhage and iron porphyrin metabolism, and two more clinical articles on the macrocytic anemias and the hemolytic mechanisms.

S.E.

A HEMATOLOGICAL AND HISTOLOGICAL STUDY OF THE BONE MARROW AND PERIPHERAL BLOOD OF THE ADULT DOG *P E Rskers and M Coulter* From the Department of Radiation Biology The University of Rochester School of Medicine and Dentistry, Rochester New York *Am J M Sc* 216 643-655 1948

These data should provide a useful baseline for those investigators studying hematologic changes in the dog. The authors demonstrate a similar differential cell distribution in different areas of marrow although the degree of cellularity was variable

C A F

## THERAPY OF ANEMIA

OBSERVATIONS ON THE ETIOLOGIC RELATIONSHIP OF ACHYLIA GASTRICA TO PERNICIOUS ANEMIA X ACTIVITY OF VITAMIN B<sub>12</sub> AS FOOD (EXTRINSIC) FACTOR *L Berk W B Castle A D Welch R W Heimle R Anker and M. Epstein* From the Thorndike Memorial Laboratory and Harvard Medical School Boston Massachusetts and from the Departments of Medicine and Pharmacology, Western Reserve School of Medicine Cleveland Ohio New England J Med 239 911-913, 1948

The Boston investigators had previously demonstrated a heat stable food substance so-called extrinsic factor which in combination with a heat labile factor of normal gastric juice produces a hematopoietic response in patients with pernicious anemia. They report the potentiation of crude and concentrated liver extracts by gastric juice as well. This article is concerned with the action of normal gastric juice on four patients given 5 gamma of vitamin B<sub>12</sub> daily by mouth. In each a greater reticulocyte rise occurred with the simultaneous administration of gastric juice and B<sub>12</sub>. It is suggested that the extrinsic factor may be identical with or closely related to the antipernicious anemia factor of liver which itself is presumably identical with B<sub>12</sub>. Since patients with pernicious anemia have normal amounts of B<sub>12</sub> in their feces it appears that the function of normal gastric juice is to facilitate its absorption

C A F

PTEROYLGLOUTAMIC ACID AND RELATED COMPOUNDS *T H Jukes and E L R Stokstad* From the Lederle Laboratories Division American Cyanamide Co, Pearl River New York. *Physiol Rev* 28 51-106 1948

This review of folic acid knowledge delves into extensive historical data on vitamin M, vitamin B<sub>9</sub>, xanthopterin, orotic eluate factor and *Streptococcus lactis* R factor and then discusses the analysis synthesis and properties of pteroylglutamic acid. The role of the material in the nutrition of various animals (mice dogs guinea pigs pigs mink insects) is covered and the interrelationships of conjugated forms. There is a closing section the clinical uses of the drug

S E

FOLIC ACID Editor *R W Miner* From the Museum of Natural History N Y Ann New York Acad Sc 48 255-350 1948

This monograph details information on the history chemistry and pharmacology of folic acid and presents a few notes on its clinical effects in pernicious anemia and nutritional macrocytic anemia. The data cover the knowledge of the substance up to November 1948

S E

PTEROYLGLOUTAMIC ACID DEFICIENCY IN MICE HEMATOLOGIC AND HISTOLOGIC FINDINGS *D R Weir R W Heimle and A D Welch* From the Departments of Medicine and Pharmacology School of Medicine Western Reserve University and the University Hospitals of Cleveland Cleveland Ohio *Proc. Soc Exper Biol & Med* 69 211-215 1948

Maintained from the time of weaning on a pteroylglutamic acid (PGA) deficient diet and a PGA antagonist later supplemented with succinylsulfathiazole mice developed granulopenia lymphopenia, and anemia in 30-60 days. Histologic examinations of the spleen at this stage indicated practically complete cessation of normal hematopoietic activity and excessive hemosiderin deposits in that organ. Simultaneous bone marrow changes are described indicating marked hyperplasia of immature elements identified as primitive blastic cells and a depletion of more adult forms interpreted as evidence of maturation arrest. Following the parenteral administration of PGA the regimen being otherwise unaltered the characteristics of the peripheral blood spleen and marrow promptly reverted to normal. Thus whereas



the vitamin may not be required in mice for the formation of the most immature blood elements the maturation of these cells apparently depends on the presence of PGA

C.P.E.

**FAILURE OF XANTHOPTERIN TO INFLUENCE HEMATOPOIESIS AND GROWTH IN RATS** *J A Pritchard* From the Department of Pharmacology School of Medicine Western Reserve University Cleveland, Ohio *Proc. Soc. Exper Biol. & Med.* 69 221-225 1948

The effects of pteroylglutamic acid (PGA) and of xanthopterin were studied in weanling female albino rats maintained on a purified folic acid-deficient diet supplemented in one series by sulfathiazole and, in another, by succinylsulfathiazole a portion of the latter group being subjected to repeated bleedings in order to produce anemia. The majority of sulfathiazole treated rats developed anemia regarded as hemolytic in origin, accompanied by evidences of reticulocytosis leukocytosis and increased excretion of bile pigments not completely correctible by PGA or xanthopterin. Those receiving succinylsulfathiazole and in which blood loss anemia was artificially imposed, exhibited erythrocytic, leukocytic and growth responses when PGA was supplied xanthopterin proving completely inert.

C.P.E.

**A METHOD FOR STUDYING THE EFFECT OF VARIOUS SUBSTANCES UPON RED CELL MATURATION IN VITRO** *E E Hays* From the Department of Biochemistry, University of Vermont College of Medicine, Burlington Vermont *Am J. M. Sc.* 216 528-533 1948

The author describes in more detail his method of assaying hematopoietic factors in vitro. The bone marrow cells of rats are used and the number of reticulocytes counted after a period of incubation with various substances. His studies would indicate that normal rat serum, human serum, liver extracts potent in treatment of pernicious anemia,  $B_{12}$ , Tyrosine, glutathione and Bacto-yeast extract appear to have a red cell maturing effect, i.e., a high reticulocyte count in the incubated marrow. Folic acid was not active.

In vitro methods of studying hematopoiesis are needed. This method, while perhaps a rough index of alteration in the marrow cells, has the advantage of being simple and has been shown by the author in other studies to be a worthwhile assay method for potency of liver factors.

C.A.F.

**TREATMENT OF IDIOPATHIC PERNICIOUS ANEMIA** *R L Haden and D W Bortz* From the Cleveland Clinic and the Frank E. Bortz Educational Institute Cleveland, Ohio *J. A. M. A.* 138 870-873, 1948

This is a general review article which emphasizes that the necessary and sufficient therapy in pernicious anemia is refined parenteral liver extract and that all other drugs (pteroylglutamic acid, iron hydrochloric acid, vitamins, oral preparations of liver) are useless and unnecessary. There is no discussion of vitamin  $B_{12}$ .

S.E.

## LEUKOCYTES, LEUKEMIA AND LYMPHOMA

**A STUDY OF THE BLOOD OF SOME CRUSTACEA** *W C George and J Nichols* From Department of Anatomy, the University of North Carolina Chapel Hill North Carolina. *J. Morphol.* 83 425-443 1948

The blood of most crustacea contains two main classes of cells when examined in the living condition, viz. those with refractile granules or globules and those without. Careful examination with higher magnification reveals that only a few have a truly hyaline cytoplasm. In general four types of blood cells are recognizable. Lymphoid cells which are few in number, small and globular or spindle shaped. The second type are thigmotactic amoebocytes, which are semi hyaline and sometimes finely granular. These cells are the most active phagocytes of the blood and they can ingest India ink when injected into the animal. A third type consists of those cells with coarse, refractile and acidophilic granules. The fourth type of cell contains refractile granules which are intermediate in size between the fine granules usually seen in semi hyaline thigmocytes and the cells with coarse refractile granules. Type I is comparable in the mammalian lymphoblast or hemocytoblast and type II to the monocyte or free macrophage. Hematogenesis occurs in the blood channels. The clotting mechanism is powerful in some crustacea, weak in others.

O.P.J.

**MAST CELLS THEIR DISTRIBUTION IN VARIOUS HUMAN TISSUES** *J. Janis and J. R. McDonald* From the Mayo Clinic, Rochester, Minnesota Arch Path 45 622-634, 1948

Tissue mast cells have been the objects of many researches, but it took the work of Holmgren and Wilander (1937) to focus our attention on their probable physiologic role with respect to heparin production. This has stimulated investigators to apply various physical and histochemical techniques in order to ascertain the nature of the metachromatic granules in these cells. Wislocki and Dempsey (Anat Rec 96 249, 1946) studied mast cells in the human and rat and found a species difference with respect to the presence of lipoidal material. The basophilic granules were not digested by ribonuclease or hyaluronidase. Noback and Mootagna (Anat Rec. 96 279 1946) showed that alkaline phosphatase was localized in the majority of mast cell granules. The cytochrome C-cytochrome oxidase system was also present. Wislocki, Bunting and Dempsey (Am J Anat 81 1, 1947) showed that the metachromatic reaction was unaltered by hyaluronidase and that the granules did not give the Baner reaction after digestion with saliva. Mootagna and Noback (Anat Rec 100 535, 1948) have extended our knowledge by demonstrating mast cell granules contain phospholipid peroxidase and lipase. Janis and McDonald have studied the distribution of these cells in a wide variety of human tissues and have concluded that their presence in synovial membranes may explain why the hemarthrosis is often fluid in closed injuries of joints. There is a definite increase of mast cells in the synovial membrane in tuberculosis and other chronic infections. When a solution of protamine was added to fluid blood aspirated from a knee it formed a definite coagulum. This suggests that heparin may in part be responsible for the prevention of clotting in hemarthrosis.

O.P.J.

**LEUCÉMIE MYÉLOÏDE À POLYNUCLÉAIRES ET POLYGLOBULIE (MYELOID POLYNUCLEAR LEUKEMIA AND POLYCYTHEMIA)** *P. Emile Weil and S. Perles* Sang 19 442-447 1948

Polynuclear leukemia is a type of myeloid leukemia described by P. E. Weil in 1937 (Sang 5 539 1937) with the following characteristics: moderate increase of the leukocytes (average 30 000 to 50 000), 80 to 99 per cent of these leukocytes being adult polynuclears, myelocytes very scarce (2 to 3 per cent or absent), very few metamyelocytes. Bone marrow, liver, spleen show the usual features of myeloid leukemia with more than the usual ratio of adult cells (for instance, 30 per cent myelocytes, 30 per cent metamyelocytes, 30 per cent polynuclears). The evolution of the disease usually is very slow (ten years).

Since the original description Weil has seen 15 cases of such polynuclear leukemia. The red cells are usually slightly modified and some nucleated red cells can be found in the peripheral blood. In the 2 cases reported in the present communication a polycythemia between 6 and 8 millions was observed.

The authors discuss the relation between myeloid leukemia, myeloid metaplasia of the liver and spleen and polycythemia. Numerous forms of transitions are possible between these syndromes with an initial onset of polycythemia or of myeloid hyperplasia and with a more or less malignant character of this hyperplasia.

J.P.S.

**MEGAKARYOCYTIC LEUKAEMIA WITH THROMBOCYTHAEMIA** *G. Hemmeler* Clinique medicale universitaire, Lausanne (Switzerland) Schweiz Med Wchnschr 78 967-977 1948

The case described shows as an outstanding symptom a platelet count up to 3.3 millions without anemia or leukosis. In the bone marrow enormous increase of megakaryocytes with normal differential count and morphology. Clinically good general condition, no lymphomata and only slight enlargement of spleen. Treatment with urethane, arsenic and nitrogen mustard caused a decrease of thrombocythemia and splenomegaly.

The case shows a selective increase of megakaryocytopoiesis without participation of hematopoiesis or leukopoiesis.

C.M.

**TREATMENT OF THE LEUKEMIAS** *G. L. Kant Jr* From the Department of Medicine, Cornell University Medical College, New York, New York. Am J M Sc. 216 581-595 1948

This article encompasses in its discussion therapeutic agents of theoretic and practical value in leukemia. The current literature is briefly and critically summarized (89 references).

C.A.F.

*THE NEGATIVE EFFECT OF FOLIC ACID ON IRRADIATION LEUKOPENIA IN THE CAT* *W S Adams and J S Lawrence* From the University of Rochester School of Medicine and Dentistry and the Medical Department of Strong Memorial and Rochester Municipal Hospitals Rochester New York *Am J M Sc.* 216 656-660 1948

The authors present convincing evidence that folic acid orally or parenterally did not influence the leukopenia produced in cats by exposure to 200 r whole body irradiation

C.A.F

*EXPERIMENTAL AND CLINICAL THERAPEUTIC STUDIES ON LYMPHOSARCOMA* *C P Rhoads* From Memorial Hospital New York City New York *Ann Int. Med* 29 811-821 1948

What is known of the natural history of lymphosarcoma is discussed The various advantages of surgical radiation P<sub>33</sub> mustard and other miscellaneous agents in the management are reviewed

C.A.F

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## BOOK REVIEW

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*The Rh Blood Groups and their Clinical Effects* By P L MOLLISON, A E MOURANT AND R R RACE Med Res Council #19 London, His Majesty's Stationery Office, 1948 Revised, reprinted November 1948 Pp 74 Price 1 shilling, 6 pence, net

This short booklet by three eminent British authorities summarizes present knowledge of the Rh-Hr factor and presents this information in an understandable manner The subject is roughly divided into three main headings, and each is discussed by the investigator most intimately concerned with that phase of the topic Dr Race describes the present concept of the genetic inheritance of the various Rh genes, their distribution in the population and the roles which the various factors of the Rh-Hr complex (C, D, E, c, d, e, ) play in incompatibility matings Dr Mollison takes up the clinical considerations of this subject He discusses in detail the ways in which iso-immunization to the Rh factor occurs, the role of sensitization in hemolytic transfusion reactions and in the causation of hemolytic disease of the newborn The topics of diagnosis, pathologic anatomy and therapy of this affection of the fetus are well handled and most of the disputed points and unsolved problems are clearly discussed Dr Mourant describes the actual mechanics of the various tests and manipulations now in use in the routine typing procedures in the diagnosis of sensitization and the establishing of the presence of hemolytic disease in the newborn

This booklet expounds concisely and quite clearly our present state of knowledge concerning this important subject

JACOB NEBER

# BLOOD

## *The Journal of Hematology*

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VOL IV, NO 7

JULY, 1949

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### THE CLINICAL ASSOCIATION OF MACROCYTIC ANEMIA WITH INTESTINAL STRICTURE AND ANASTOMOSIS

By D G CAMERON, M D , G M WATSON, M B , D PHIL ,  
AND L J WITTS, M D

BEFORE 1900, several Scandinavian authors had observed that intestinal stricture might be associated with an anemia resembling pernicious anemia. Faber,<sup>11</sup> who described a case of pernicious anemia in a young woman with multiple intestinal strictures, was the first to recognize the relationship between the two conditions. Other reports followed, and Meulengracht<sup>20</sup> reviewed 22 cases of macrocytic anemia associated with intestinal stricture. Little, Zervas and Trusler<sup>17</sup> described a case of anemia, with a blood picture typical of pernicious anemia, in a young man on whom a series of operations had been performed to cure a fistula, the sequel of acute appendicitis. In this case, anastomoses were present and it appears to be the first recorded in which the anemia was associated with the presence of anastomoses. This patient responded well to liver therapy and relapsed when this was discontinued. Since 1929, further cases of both stricture and anastomotic anemia have been described, though it has not always been possible to separate the two conditions. Barker and Hummel<sup>1</sup> reviewed 51 cases, 2 of their own and 49 collected from the literature. Since their publication, additional cases have been reviewed by Jensenius,<sup>16</sup> and a recent case has been reported by J. E. Richardson.<sup>22</sup>

The main features of the published cases of this anemia are analyzed in table 1. This table is based on the figures given by Barker and Hummel, but has been amended by the inclusion of cases published since their review and a further case admitted to the Radcliffe Infirmary in 1944 (R I 26019/44) which is described below. Two other cases have been reported briefly by Wintrobe<sup>3</sup> but as no details are given they have been omitted from the table. Anastomosis was the basic abnormality in 23 cases, while in 37 cases one or more strictures were present. One case had multiple diverticula. Of the anastomoses, 15 were entero-enterostomies or entero-colostomies, 2 of which had fecal fistulae in addition, and 8 were gastro-colic or high jejuno-colic fistulae. The strictures were mostly of the small intestine but 6 were in the colon. In 12 cases, the strictures were shown to be tuberculous, in 3 others regional ileitis was present or had been resected, and some cases reported as tuberculous but without definite proof, may properly belong in this category. In other cases the stricture was secondary to adhesions, and in some no cause for the stricture could be found. In some of the 8 cases where there was gastro-colic or

From the Nuffield Department of Clinical Medicine Radcliffe Infirmary Oxford England

jeuno-colic fistula the anemia may be partly attributed to an underlying lesion carcinoma or peptic ulcer. In a few cases, the intestine had been resected to a varying extent, but in no case more than 60 cm., and it is unlikely that resection was an important factor in the production of anemia, as Jensenius<sup>18</sup> has shown that much more extensive resections are necessary to produce anemia, both in man and in the experimental animal.

### CASE REPORT (R I 26019/44)

The patient was a woman of 42 whose mother was known to have pernicious anemia. In 1938 this patient first developed symptoms of subacute intestinal obstruction. After three months a laparotomy was done and adjacent loops of ileum anastomosed with relief of the abdominal symptoms. In 1939 she again became ill and was found to have a severe hyperchromic anemia with a hemoglobin value of 5.9 Gm. per cent and an erythrocyte count of 1.69 millions. The blood film showed macrocytosis, anisocytosis and poikilocytosis. Treatment at this time with an oral liver preparation was very effective.

In the summer of 1943 she was unable to obtain oral liver extract and was treated with an intramuscular preparation. In spite of intensive therapy, she began to get sore tongue and indigestion, she lost weight and the anemia recurred. She complained of abdominal discomfort and distension and of paraesthesia in the hands and feet. The stool was unformed with a bowel action usually twice a day.

She was admitted to this hospital in April 1944. There was slight clubbing of the fingers. There was extensive edema of the legs and a little glossitis. The abdomen was distended in the center by a swelling apparently composed of firm coils of bowel and there was visible and noisy peristalsis. Physical examination was otherwise negative. The blood pressure was 135/90, the urine was normal and there was no objective evidence of neurologic disease. Laboratory investigation at this time gave the following results:

<i>Blood</i>	Blood urea 22 mg. %
Hemoglobin 9.4 Gm	Plasma bilirubin 0.2 mg. %
Erythrocytes 2,630,000	Plasma phosphatase 3 units
Color index 1.2	Plasma phosphate 3.33 mg. %
Reticulocytes 1.6%	Serum calcium 7.8 mg. %
Leukocytes 3,200	Plasma cholesterol 120 mg. %
Platelets 260,000	Plasma ascorbic acid 0.2 mg. %
Hematocrit 33%	Plasma protein (total) 4.17 Gm. %
Mean cell volume 125 $\mu^3$	Plasma albumin 2.10 Gm. %
Mean cell diameter 8.24 $\mu$	Plasma globulin 1.70 Gm. %
Prothrombin time Normal	Plasma fibrinogen 0.37 Gm. %

*Sternal marrow* Smears showed an active marrow with both normoblastic and megaloblastic hemopoiesis. There were 12 per cent megaloblasts and 21 per cent normoblasts in the film.

*Fractional test meal* Free hydrochloric acid was present (this was still true in 1947).

*Barium meal* Evidence of relative small bowel obstruction with hypermotility.

*Absorption tests* Glucose and sucrose tolerance tests were within normal limits.

*Fat balance* For this test the diet contained 70 Gm. of fat daily.

	1st day	2nd day	3rd day
Total fat as per cent of dried feces	18.7	16.2	17.5
Split fat as per cent of total	91	97	99
Total fat excreted	6.36 Gm.	3.03 Gm.	6.57 Gm.

There was no occult blood in the feces.

She was treated with a low fat, high protein diet together with yeast and proteolyzed extract of liver by mouth. Plasma protein levels rose to a total of 6.1 Gm. per cent with 3.32 Gm. albumin and edema diminished. Finally, as a preliminary to operation, a transfusion of two pints of blood was given. Mr.

D C Corry operated on the patient on May 24 1944 through a right paramedian incision. The previous anastomosis was identified an inch above the ileo-caecal valve. The excluded coil of bowel, which was about two feet in length contained several strictures and the intervening musculature was greatly dilated and hypertrophied. The mesentery was thickened in a way similar to that of regional ileitis, but the bowel wall was not so rough as in regional ileitis and appeared whiter. Mr Corry resected the excluded loop and did a side-to-side anastomosis to reconstitute the bowel. Dr J R O'Brien reported as follows on the specimen:

The specimen consists of about 70 cms of small intestine, the two ends of which are honed together to form a rough circle. There are at least nine constrictions in the wall, with dilatation in between, giving a beaded effect. The largest cavity measures  $12.0 \times 7.0$  cms in diameter, and the narrowest constriction appears to be only about 0.5 cm wide. The wall is slightly thickened throughout. The average thickness is 0.4 cm while at each constriction there is considerable increase in the thickness of the wall which appears to be mainly due to muscular hypertrophy with a maximum thickness of 1.1 cms.

The mucosa appears natural except for the absence in places of rugae due presumably to the distension and there is necrosis and ulceration of the mucosa in relation to the stomata. Also in relation to these there are many polypoid outgrowths, the largest being 1.0 cm in diameter, and 0.8 cm high. This one is sessile, some are pedunculated. At one place the constriction has extended for about 4.0 cms in length while the majority of constrictions are only about 1.0 cm wide. There is considerable increase of fat in the mesentery in places particularly in relation to the stenotic areas.

Microscopically the mucosa appears to be natural but there is a very marked hypertrophy of both the muscularis mucosae and the circular and longitudinal muscles. It is the muscular hypertrophy which produces the preponderance of the increase in thickness of the wall. There is a very extensive chronic inflammatory change with a moderate amount of scarring. On top of the chronic change there is also an acute inflammatory process extending through into the muscle layer. In addition there are a large number of lymphoid aggregates scattered in the muscle layer and more particularly on the peripheral surface of the muscle in the attachment of the mesentery. There are areas of early calcification in the muscle layer. The microscopic picture is in fact disappointing, the predominant features being the acute inflammatory process with polymorphs extending into the muscle layer, the muscular hypertrophy and a background of chronic inflammation.

After operation the patient made a good recovery in all respects. Diarrhea was a little troublesome at first, but it responded to treatment with syrup of codeine and prepared chalk and by the end of two months the bowel rhythm had been re-established at one motion a day without medication. It was still necessary however for her to be careful with her diet and to avoid fresh fruit, salads and coarse vegetables. She rapidly gained weight. Parenteral liver extract was continued until October, 1944 when she became sensitized to liver and had severe reactions after the injections. At this time the blood count was red cells 5.03 million per cu mm, hemoglobin 13.2 Gm per cent, color index 0.9, white cells 7,600. The Price Jones curve which had been displaced to the right had come back to normal. The plasma proteins were 6.35 Gm per cent with 4.2 Gm albumin. It was decided to see the effect of discontinuing liver.

The patient remained well for a little less than a year. There was then a sharp relapse and in October 1945 the hemoglobin had fallen to 5.6 Gm per cent. She was therefore desensitized to liver and intramuscular treatment was resumed. All went well again until the beginning of 1947 when her father died. The patient, who is a rather emotional woman, collapsed after this and later had an illness which was called gastric influenza.

She was readmitted to hospital on April 24 1947. She had lost about 10 kilos in the last six months and there was slight clubbing of the fingers. Physical examination was otherwise negative. The stools showed no gross abnormality on microscopy or culture and no occult blood. There was moderate anemia, red cells 4,400,000 per cu mm, hemoglobin 11.6 Gm per cent, mean corpuscular volume  $99 \mu^3$ , white cells 7,100, ESR 9 mm. The plasma proteins totalled 5.1 Gm per cent with 3.0 Gm albumin. Free acid was present in the test meal in high normal concentrations. On x-ray examination there was practically no gas in the abdomen. The small bowel was much shorter than normal and the terminal loops showed a moderate degree of dilatation. There were changes in the pattern which strongly suggested an extensive recurrence of the original lesion.

Further surgery was not advised and she resumed treatment with a low residue, high protein diet.

extra vitamins and intramuscular liver extract. By May 1948 she had regained 6 kilos and was relatively free from symptoms.

### DISCUSSION

In the present patient, anemia developed after anastomosis had been performed to short-circuit a stricture due to regional ileitis, and its persistence after resection of the bypassed loop appears to be due to an extension of the disease process up the small intestine. In view of the repeated finding of free hydrochloric acid in the gastric juice, and the failure of the anemia to respond to intramuscular liver therapy until the anastomosis had been corrected, it is unlikely that the case represents the fortuitous association of Addisonian anemia with gross intestinal disease. Nevertheless, it is of interest that the patient's mother had pernicious anemia and an inherited predisposition may have been a factor. A further point worth making about this woman is that there was no obstruction to the passage of food along the small intestine, stagnation was confined to the bypassed loop.

TABLE 1—*Analysis of 61 Cases of Intestinal Stricture or Anastomosis Associated with an Anemia Resembling Pernicious Anemia*

	Present	Absent	Not recorded
Gastro intestinal symptoms	52	2	7
Glossitis	33	6	22
Neurologic disease	12	19	30
Icterus	19	19	23
Macrocytosis	49	1	11
Hyperchromia	41	11	9
Poikilocytosis	24	—	37
Leukopenia	35	7	19
Free HCl in gastric juice	24	20	17

The main features of the macrocytic anemia of intestinal stricture and anastomosis have been discussed by Barker and Hummel and by Jensenius, and they are summarized in table 1. In the 11 cases where the presence of macrocytosis has not been recorded, the blood picture has been described only as resembling that of pernicious anemia. In general, the blood picture in these anemias closely resembles that of pernicious anemia in that hyperchromia, macrocytosis and anisocytosis are prominent features. Poikilocytosis also is often prominent, in most of the cases where its presence has not been recorded, there has been no detailed description of the blood. In some cases, however, it has been noted that poikilocytosis has been slight and less than might be expected in pernicious anemia of the same degree.<sup>1 4 5 12 19</sup> These reports include 4 cases of stricture anemia and 4 of gastro-jejuno-colic fistula. In the macrocytic anemia of sprue, poikilocytosis is less prominent than in pernicious anemia. In the present series of cases, there are 9 in which steatorrhea has been demonstrated by analysis of the stool, in 4 of these, poikilocytosis was recorded as slight, in the other 5 there is no record. Nucleated red cells have been seen occasionally in peripheral blood films, and in three cases<sup>14 17 23</sup> megaloblasts have been reported.

### *The Bone Marrow*

For most of the cases, there is either no record of the bone marrow or it has simply been described as red and hyperplastic. In 8 cases, megaloblasts have been reported in the marrow. Zadek<sup>23</sup> found megaloblasts in the marrow in his two cases, Hartmann<sup>27</sup> in his, Fairley and Kilner<sup>12</sup> in their third case, Hawksley and Meulengracht<sup>15</sup> in their case, Barker and Hummel<sup>1</sup> in their 2 cases and the sternal marrow was megaloblastic in our own case. In one case, Zadek found only 1 per cent of megaloblasts in sternal marrow smears. In Barker and Hummel's first case, the sternal marrow film was said to resemble that of pernicious anemia but contained only 2.5 per cent of megaloblasts. After liver therapy, it became normal. Fairley and Kilner remark that in their case the megaloblastic transformation was far from complete. Evidently a megaloblastic transformation may be seen in the marrow similar to that of pernicious anemia, but the change may be to a lesser degree. In one case,<sup>10</sup> the marrow was aplastic, infection was present and this patient did not respond to liver therapy.

### *The Effect of Liver Therapy*

The effect of liver therapy in these cases has been reviewed by Barker and Hummel<sup>1</sup> and Jensenius.<sup>16</sup> Liver therapy was used in 27 of the 61 cases reviewed here, in 5 of these, it was without effect and in the remaining 22 a response was obtained. This response varied considerably in degree, being often less than might be expected in pernicious anemia of comparable severity. In 8 of these cases, oral administration of a liver preparation was effective, and it is important to note that in 5 cases, parenteral therapy was effective where oral administration had failed. Treatment with liver seems to have been less certain in its effect than in true pernicious anemia but it should be remembered that some of the patients who derived little benefit from liver therapy were extremely debilitated or had some complicating lesion such as carcinoma. In 4 of the 5 cases where liver therapy produced no response at all, the patients were moribund, and the fifth had active pulmonary tuberculosis from which he died after operation.

### *The Effect of Surgical Correction of the Intestinal Lesion*

The direct relation of the anemia to the intestinal lesion has been shown by the fact that, in several cases, surgical correction of the intestinal abnormality has led to cure of the anemia. In this series, operative treatment was carried out in 25 cases. The results of operation are shown in table 2. Fifteen of the cases had liver therapy in addition to surgical treatment. The mortality appears high, but in many cases the technical difficulties were considerable, and in others the presence of carcinoma as the underlying cause made success improbable. The earliest cases were undertaken before liver therapy and blood transfusion were available to improve the preoperative condition. The importance of a high protein diet to repair the hypoproteinemia and edema is also better recognized today.

The cases which survived operation will be described briefly. Sturgis and Goldhamer<sup>20</sup> (case 7) reported a case of ileo-cecal fistula with macrocytic anemia, in which an attempt was made to correct the fistula, but this was unsuccessful.



In 5 cases, operation was of no benefit or produced only a temporary remission of the anemia. Little, Zerfas and Trusler<sup>17</sup> described a case of anemia in association with two jejunal anastomoses. These were undone and 25 cm. of distended jejunum excised. Improvement followed but the anemia later recurred. Another case was noted by Bethell<sup>2</sup>, here details are lacking but resection of a stricture gave relief from a macrocytic anemia, without liver therapy, for 8 months relapse then followed the formation of fresh adhesions. The 3 remaining cases in which operation did not lead to cure of the anemia were all diagnosed as regional ileitis, and it is to be expected that the lesion would recur (Barker and Hummel,<sup>1</sup> case 1, Sturgis and Goldhamer,<sup>20</sup> case 6, and our own case). It is interesting that in the case reported by Barker and Hummel an anastomosis performed to bypass a stricture made the anemia worse.

The first case in which operation was successful was reported by Seyderhelm,<sup>23</sup> excision of a stricture curing the anemia. Another case of stricture anemia was described by Scherer.<sup>24</sup> In this instance, ileo-colostomy was performed to bypass a tuberculous stricture of the ileum, marked improvement followed during a short observation period. Butt and Watkins<sup>6</sup> described a similar case in which ileo-

TABLE 2.—*The Results of Surgical Correction*

	Number of cases
Death from operation	11
Operation technically unsuccessful	1
Failure to correct anemia	5
Cure of anemia	8
	—
Total cases	25

colostomy was performed for terminal ileitis, the operation was claimed to cure the anemia but the patient was not followed up. In these 2 cases, prolonged observation might have shown that the improvement was not maintained. Cases of gastro-jejuno-colic fistula with accompanying macrocytic anemia which was cured by surgical correction of the fistula have been reported by Fairley and Kilner<sup>1</sup> (case 1) and Bennett and Hardwick.<sup>2</sup> Christopher<sup>8</sup> described a case of macrocytic anemia in association with multiple anastomoses between the ileum and the colon, surgical correction of the intestinal lesions led to cure of the anemia, and liver therapy was not needed. W. Richardson<sup>25</sup> reported a case of macrocytic anemia in a young man in whom an entero-enterostomy had been performed as a sequel to acute appendicitis. This patient responded partly to liver therapy but jaundice remained. At a further operation it was found that about half the small intestine had been short circuited, this was corrected and the patient became completely well and needed no further liver. In a case reported by J. E. Richardson,<sup>22</sup> a high jejuno-colostomy was performed after appendicitis, and this patient developed a macrocytic anemia. Surgical correction led to complete cure.

From these results it is clear that where it has been practicable to correct the intestinal abnormality the anemia has been cured or greatly improved. In 4 of the

5 cases in which operation was unsuccessful, or produced only a temporary remission, there was an underlying abnormality which was progressive and could not be permanently eradicated by surgical measures

### *The Relation of Steatorrhea to the Macrocytic Anemia*

Macrocytic anemia may be found in other intestinal disorders, notably the steatorrheas, and as steatorrhea has been found in some of the cases reviewed here it is necessary to consider whether the macrocytic anemia of stricture or anastomosis might not arise from the presence of steatorrhea rather than directly from the intestinal lesions

In 10 of the 61 cases, the fat content of the feces has been estimated, 9 of these providing evidence of steatorrhea. In 2 other cases,<sup>1</sup> blood fat levels have been followed after ingestion of a fatty meal. In 2 cases, the stools<sup>2</sup> have been described

TABLE 3—*Steatorrhea and Macrocytic Anemia*

Author	Diagnosis	Fecal fat figures	
		Total fat %	
Fairley and Kilner <sup>12</sup>	Gastro-jejuno-colic fistula	33.9	(dry)
Fairley and Kilner <sup>12</sup>	Gastro-jejuno-colic fistula	47	(dry)
Fairley and Kilner <sup>12</sup>	Gastro-jejuno-colic fistula	31.8	(dry)
Hawksley and Meulen gracht <sup>15</sup>	Tuberculous strictures of intestine	51	(dry)
Mindline and Rosen heim <sup>21</sup>	Duodeno-colic fistula	92	(dry)
Brock <sup>1</sup>	Multiple strictures of intes- tine	28 Gm } 7 Gm } 27 Gm } 13 Gm }	4 day period    Diet 45 Gm daily
Salvesen and Kobro <sup>24</sup>	Gastro-jejuno-colic fistula	7	(moist)
Salvesen and Kobro <sup>24</sup>	Stricture of middle gut	15.3	(moist)
Bennett and Hardwick <sup>2</sup>	High jejuno-colic fistula	56	(dry)

as resembling those of sprue,<sup>9</sup> - in one case the stool was said not to be fatty,<sup>11</sup> and in another the stool is said not to have been that of sprue.<sup>25</sup> In the remaining cases, there is no specific information on the nature of the stool. In some of these cases, the stool has been noted to be offensive or pale, and steatorrhea may have been present, in most cases the presence of diarrhea, often watery, has been mentioned, and it seems likely that the majority did not have steatorrhea. Table 3 shows the cases in which laboratory evidence of steatorrhea is available.

From table 3 it is seen that 6 of the 9 cases in which there was positive evidence of steatorrhea were patients with gastro-jejuno-colic, duodeno-colic or high jejuno-colic fistula. There are 3 further cases of this description in the series, in one of these,<sup>9</sup> a diagnosis of sprue had been made originally, and in another<sup>2</sup> the stools were like those of sprue. In the third<sup>1</sup> (case 2) the blood fat curve after ingestion of a fatty meal was normal, but this method cannot be relied upon to detect abnormalities of fat absorption. It is clear that cases with gastro-colic or other high

anastomoses usually have steatorrhea, but steatorrhea has been demonstrated in only 3 of the remaining 52 cases of stricture or anastomosis, though it may have escaped observation in some of these. Our own case, in which the fecal fat output over a three-day period on a constant diet was within the normal range, demonstrates that steatorrhea is not essential to the development of macrocytic anemia in these cases. Further evidence is provided by the 2 cases, referred to above, in which the macroscopic appearance of the stool did not suggest steatorrhea. It was noted earlier that poikilocytosis, which is not a feature of sprue, was less in evidence in the cases in this series with steatorrhea, and the anemia of these cases may resemble that of sprue rather than that of pernicious anemia. Jenseni<sup>16</sup> considers that stricture anemia resembles pernicious anemia very closely, but that anastomotic anemia resembles spure. This appears to be the case only where there is a gastro-colic or high jejuno-colic fistula.

### *Pathogenesis of Stricture Anemia*

Pernicious anemia is a disease of the latter half of life which, if untreated, follows a remittent course to a fatal ending. It is characterized by a severe macrocytic and hypochromic anemia, megaloblastic change in the bone marrow, leukopenia and a therapeutic response to liver extract. In addition to abnormalities of blood formation there is evidence of increased blood destruction. The gastric secretion as a whole is reduced and achlorhydria is almost invariably present. The gastric mucosa is atrophied, this change may involve more of the alimentary tract and glossitis is common. Pathologic changes in the central nervous system are frequently present.

It is clear that most the features of pernicious anemia, given in this brief description, are present in the macrocytic anemia associated with intestinal stricture or anastomosis. Though there are some important differences the similarity is such that it is probable that the two conditions are closely related, and that the abnormal hemopoiesis present in both has a common origin. That the occurrence of macrocytic anemia with intestinal lesions does not represent the fortuitous association of the two diseases is shown by several points. The age distribution differs from that of pernicious anemia in that younger subjects are equally susceptible, free hydrochloric acid is frequently present in the gastric juice and intrinsic factor has been shown to be present in one case,<sup>36</sup> although it was absent in the only other case in which its activity was investigated.<sup>7</sup> Finally, there is the point that the anemia may be cured by surgical correction of the intestinal abnormality.

With regard to the pathogenesis of stricture anemia, Faber<sup>11</sup> originally postulated the absorption of a poison from the stagnant bowel content. Meulengracht<sup>18</sup> held the same view and considered it to support the theory of the intestinal origin of pernicious anemia, while Seyderhelm<sup>23</sup> went so far as to practice, with some success, ileostomy and lavage for pernicious anemia. After Castle's work, these views needed some modification and Barker and Hummel<sup>1</sup> considered various possibilities. It might be thought that stagnation interferes with the absorption of hemopoietic principles, but these authors found that, in general, absorption tests in these anemias were usually good. Lack of extrinsic factor in the diet or the presence of disease of the liver could not be incriminated. They concluded that the liver prin-

ciple did not act directly on the bone marrow but promoted detoxification of compounds of intestinal origin which might cause harmful changes throughout the body. It seems more probable that those compounds act by interfering with the formation, absorption or utilization of materials necessary for normal erythropoiesis.

It seems established that macrocytic anemia of intestinal origin is different from Addisonian pernicious anemia inasmuch as the secretion of hydrochloric acid and intrinsic factor by the stomach may be normal. There need be no steatorrhea. The only essential factor is stagnation, whether from intestinal stenosis or a stagnant loop. Acting on this hypothesis, we have produced blind loops in the small intestine of rats and have shown that if these loops are designed so as to be filled by peristalsis, a macrocytic anemia develops in a high proportion of the animals.<sup>31</sup> This anemia is usually fatal, but in some of the rats it has responded to treatment by injection of refined liver extract. The results of further experiments will be reported in subsequent papers.

#### SUMMARY

1. The case is reported of a woman in whom a macrocytic anemia developed after a short-circuit operation for regional ileitis. At a second operation, multiple constrictions and distentions were present in the bypassed loop.

2. The literature of macrocytic anemia associated with intestinal stenosis and anastomosis is reviewed. The anemia differs from Addisonian pernicious anemia in that the gastric secretion of hydrochloric acid and intrinsic factor may be normal. There need be no steatorrhea. It is concluded that the anemia is probably due to stagnation of intestinal contents and the absorption of toxic substances.

3. The production of blind loops in the small intestine of experimental animals offers a promising approach to the study of the macrocytic anemias.

#### REFERENCES

- <sup>1</sup> BARKER W H AND HUMMEL L E. Macrocytic anemia in association with intestinal strictures and anastomoses. *Bull. Johns Hopkins Hosp.* 64: 215, 1939.
- <sup>2</sup> BENNETT T I AND HARDWICK, C. Chronic jejuno-ileal insufficiency. *Lancet* 2: 381, 1940.
- <sup>3</sup> BETHELL F H. Unpublished, cited by Barker and Hummel.<sup>1</sup>
- <sup>4</sup> BROCK J F. Intestinal stricture and megalocytic anemia. *Lancet* 1: 72, 1939.
- <sup>5</sup> BUTT H R AND WATKINS C H. Occurrence of macrocytic anemia in association with lesions of the bowel. *Ann. Int. Med.* 10-1: 222, 1936.
- <sup>6</sup> ——— and ———. Unpublished, cited by Barker and Hummel.<sup>1</sup>
- <sup>7</sup> CASTLE W B, HEATH C W AND STRAUSS M B. Observations on the etiologic relationship of achylia gastrica to pernicious anemia. *Am. J. M. Sc.* 182: 741, 1931.
- <sup>8</sup> CHRISTOPHER F. Vicious jejuno-colostomy with chronic ileac obstruction. *Am. J. Surg.* 29: 124, 1935.
- <sup>9</sup> DE RIVAS D. Abstract of discussion on sprue. 81st annual session of the A. M. A., Detroit. *J. A. M. A.* 85: 1964, 1930.
- <sup>10</sup> EMILE WEIL, P. STIEFFEL, R. AND BOUSSER J. Un cas d'anémie grave aplastique au cours de sténoses intestinales. *Sang* 7: 540, 1933.
- <sup>11</sup> FABER K. Perniciöse Anämie bei Dünndarmstricturen. *Berlin klin. Wchnschr.* 34: 643, 1897.
- <sup>12</sup> FAIRLEY N H AND KILNER T P. Gastro-jejuno-colic fistula with megalocytic anaemia simulating sprue. *Lancet* 2: 1335, 1931.
- <sup>13</sup> HARTMANN E. Hyperchrome Anämie bei Stagnation des Darminhaltes in einer Seitentasche des Dünndarms. *klin. Wchnschr.* 2: 1359, 1909.

- <sup>16</sup> HAWKESLEY J C, AND MEULENORACHT, E Intestinal stricture and its association with pernicious anaemia *Lancet* 2, 124 1936
- <sup>16</sup> JENSENIUS H Results of Experimental Resection of the Small Intestine on Dogs Copenhagen 1945
- <sup>17</sup> LITTLE W D ZERFAS, L G, AND TRUSLER H M Chronic obstruction of the small bowel *J A M A* 93 1290 1929
- <sup>18</sup> MEULENORACHT E Darmstriktur und perniziöse Anämie *Arch f Verdauungskr* 28 216 1921
- <sup>19</sup> ——— Dünndarmstrikturen und perniziöse Anämie *Darmresection Acta Med Scand* 56 432, 1922.
- <sup>20</sup> ——— Pernicious anaemia in intestinal stricture *Acta Med Scand* 72 231, 1929
- <sup>21</sup> MINDLINE J AND ROSENHRIM M. L Duodeco-colic fistula simulating idiopathic steatorrhoea *Lancet* 2, 764 1935
- <sup>22</sup> RICHARDSON J E Addisonian anaemia following enteroanastomosis *Brit J Surg* 33 71 1946
- <sup>23</sup> RICHARDSON W Pernicious anemia due to entero-enterostomy *New England J Med* 218 374 1938
- <sup>24</sup> SALVESEN H M AND KOBRO, M Symptomatic sprue *Acta Med Scand* 162 277 1939
- <sup>25</sup> SCHERER E Hyperchrome Anämie und Dünndarmstriktur *Klin Wchnschr* 9 790 1930
- <sup>26</sup> SCHLESINGER A Nachweis des Antiperniciosa Prinzips in Magensaft einer Patientin mit perniziös anämischen Blutbild bei Dünndarmstenose *Klin Wchnschr* 12 298 1933
- <sup>27</sup> SCHMIDT, W Dysfunktion des Intestinaltrakts und perniziöse Anämie *Zeitschr f klin Med* 116 337 1927
- <sup>28</sup> SEYDERHELM R. Die Pathogenese der perniziösen Anämie *Ergbn d inn Med u Kinderheit* 21 361 1922.
- <sup>29</sup> ——— LEHMANN W AND WICHELIS P Experimentelle intestinale perniziöse Anämie beim Hund *Klin Wchnschr* 2 1439 1924
- <sup>30</sup> STUKORS, C C. AND GOLDRAMER S M Macrocytic anemia other than pernicious anemia associated with lesions of the gastrointestinal tract *Ann Int Med* 12-2 1245 1939
- <sup>31</sup> WATSON G M, CAMERON D G AND WITTS L J Experimental macrocytic anaemia in the rat *Lancet* 2, 404 1948
- <sup>32</sup> WINTROBE M M Clinical Hematology London 1946
- <sup>33</sup> ZADEK I Darmulcera und Strikturen bei perniziöse Anämie *Munch med Wchnschr* 73 2165, 1926

## THE EXPERIMENTAL PRODUCTION OF MACROCYTIC ANEMIA BY OPERATIONS ON THE INTESTINAL TRACT

By D G CAMERON, B Sc, M D, G M WATSON, D Phil, M R C P, AND  
L J WITTS, M D, F R C P

THE EFFECTS of partial or total gastrectomy on the blood have been studied by many workers. The essential finding is that it is impossible to produce pernicious anemia by this means in any of the species of animals which have been used. Petri and Jensenius<sup>6</sup> have analyzed the results, and the data in table 1 are derived from their paper. They give the incidence and type of anemia after gastrectomy in experiments performed to that date. In none of the experiments has clear-cut pernicious anemia resulted, though in a few cases the blood picture has suggested that disease. Bence<sup>5</sup> reported that after two years gastrectomized pigs developed a macrocytic hyperchromic anemia with a hyperplastic bone marrow of embryonic character showing megaloblasts, his conclusions are not altogether borne out by his data, and his microphotographs do not show either a true megaloblastic reaction in the marrow or a marked macrocytosis in the peripheral blood. Geiger et al.<sup>12</sup> reported that a gastrectomized pig developed macrocytosis and hyperchromia, but they did not specify any anemia. In the rat, gastrectomy produces a microcytic hypochromic anemia.<sup>16</sup> Gastrectomized animals do not develop pernicious anemia if they become pregnant.<sup>7, 10, 18</sup> In no animal has liver been shown to be of value for the anemia of gastrectomy. In spite of these negative results, it has been shown repeatedly that in gastrectomized pigs the liver content of hemopoietic principle steadily decreases and is finally lost.<sup>4, 12, 14</sup>

The results of gastrectomy alone suggested that either the animals used have a hemopoietic system differing from that in man, or Castle's intrinsic factor is secreted in other parts of the alimentary tract as well as in the stomach. For this reason, attention was directed to the duodenum. Sharpe et al.<sup>16</sup> had shown that dried duodenum was effective in the treatment of pernicious anemia, and Meulengracht<sup>22</sup> had shown that the anti-anemic activity of the pig's stomach was greatest in preparations made from the pylorus. This, together with the histologic similarity of the Brunner glands of the duodenum and the glands of the pylorus, suggested that a pyloric gland organ was responsible for the secretion of intrinsic factor.<sup>24</sup> The distribution of the anti-anemic activity in the pig has since been shown to differ from that in man, in whom it is localized in the acid-producing portion of the stomach.<sup>11</sup>

Experiments to determine the effect of resecting the duodenum and pyloric gland organ have been carried out by several groups of workers. In some early experiments unrelated to this theory, Stasoff<sup>27</sup> had found anemia after duodenectomy while Mann and Kawamura<sup>28</sup> did not observe it. Aron and Bauer<sup>1</sup> removed the duodenum and the greater part of the pancreas from a dog and found a pro-

From the Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Oxford, England.

This investigation was in part aided by grants from the Medical Research Council and the Nuffield Foundation.

nounced hyperchromic anemia, though the animal survived only twelve days Hauswirth and Silberstein<sup>15</sup> performed pyloroduodenectomy on 8 dogs, reporting their results again after two years observation <sup>16</sup> In dogs which died a few weeks after operation there was a blood picture resembling that of pernicious anemia, with hyperchromia, anisocytosis and polychromasia, though liver therapy was without effect The remaining animals showed an anemia with fluctuating characteristics Goodman et al <sup>14</sup> excised the stomach and duodenum from pigs and found that only a mild hypochromic anemia resulted, although the liver from these animals lost its anti-pernicious anemia potency even when the duodenum

TABLE 1—*The Incidence of Anemia after Experimental Gastrectomy*

Species	Number of animals	Hypo or normochromic anemia	Hyperchromic anemia
<i>Dog</i>			
Total gastrectomy	80	62	—
Isolated stomach *	11	3	—
Partial gastrectomy	17	10	—
Elective gastrectomy†	19	19	—
Total	127	94	—
<i>Pig</i>			
Total gastrectomy	38	30	—
Elective gastrectomy	4	—	3
Total	42	30	3
<i>Monkey</i>			
Total gastrectomy	7	7	—
<i>Rat</i>			
Total gastrectomy	67	67	—

\* Shortcircuiting of stomach with provision for external drainage.

† Resection of fundus or pylorus alone

alone was resected Malmros<sup>21</sup> performed duodenectomy alone on one dog and reported a microcytic hypochromic anemia

More detailed experiments on resection of the stomach and duodenum in dogs were undertaken by Petri and his collaborators. <sup>23</sup> Three types of resection were undertaken In the first they removed the whole of the stomach and the first 2 centimeters of the duodenum, which was considered to include all the Brunner glands In the second type the pylorus and 2 centimeters of duodenum were removed, and in the third the pylorus and the whole of the duodenum All three operative procedures brought about a pellagrous and usually fatal condition accompanied by an irregular anemia of variable characteristics Macrocytosis, often transient, was seen in some animals In no case could the anemia be described as pernicious

These failures led to even more extensive resections of the gastrointestinal

tract It had been reported that both the small intestine and the large intestine possessed anti-anemic activity,<sup>2 40</sup> though these reports were later criticized<sup>10</sup> Bussabarger et al.<sup>7</sup> in addition to gastrectomy, resected 150 and 180 cm of intestine respectively from two dogs, only moderate hypochromic anemia resulted Bachrach and Fogelson,<sup>2</sup> in 7 dogs, resected the distal seven-eighths of the stomach, the duodenum, and about 30 centimeters of jejunum, which they estimated to contain the whole of the Brunner gland area, but except for a temporary post-operative anemia the animals remained healthy After a similar operation Wintrobe et al.<sup>43</sup> found a microcytic anemia The importance of the ileum was examined by Petri et al.<sup>29</sup> who, again working with dogs, resected the pylorus, 2 centimeters of duodenum, and the distal two-thirds of the small intestine Their object was to resect not only the site of formation of the intrinsic factor, but an essential part of the area to which it is reasonable to refer the interaction of the intrinsic and extrinsic factors and the absorption of the resulting hemopoietic principle These dogs developed the same signs of malnutrition which the authors had found in more limited resections An anemia was present which was macrocytic in two cases The bone marrow was normal or hypoplastic, and pernicious anemia did not develop

Some experiments have been limited to resection of the small intestine alone Miller and Rhoads<sup>45</sup> reported that excision of the greater part of the dog's ileum produces only a mild hypochromic anemia Bence<sup>5</sup> resected three meters of ileum and jejunum from a pig which subsequently became polycythemic Brown<sup>6</sup> observed the effect of excision or shortcircuiting of nearly the whole of the small intestine of the pig Excision did not lead to anemia, but when the greater part was bypassed a severe microcytic anemia was found The remaining experiments on resection of the small intestine have been reported by Petri et al.<sup>27 30</sup> and Jensenius.<sup>19</sup> These workers have studied the effect of resecting or shortcircuiting approximately two-thirds of the dog's intestine, both proximal and distal resections were tried In all cases a deficiency syndrome appeared which they have called enteroprival sprue In the majority, anemia appeared as a terminal phenomenon, it was often macrocytic but not usually hyperchromic Liver therapy was ineffective and, except in one case, the bone marrow was hypoplastic Except for the presence of macrocytosis this condition has no resemblance to pernicious anemia

With these experiments attempts to produce pernicious anemia by resection of the gastrointestinal tract would seem to have reached their practical limit In no case have they been successful and where the resection is very extensive, deficiencies other than of the hemopoietic factors must complicate the picture It is true that macrocytosis and occasionally hyperchromia, which are features of pernicious anemia, have been observed, but in the majority of cases the bone marrow has been hypoplastic, and aplastic anemia is often characterized by macrocytosis

The association of pernicious anemia with stricture and other lesions of the small intestine has been known for some time,<sup>2 9 19</sup> and there have been attempts to reproduce these conditions in experimental animals The best known are those of Seyderhelm,<sup>23 24</sup> in which strictures were formed by strips of aponeurosis fixed around the small intestine just above the cecum Seven dogs survived this operation, of these, two developed a severe macrocytic and hyperchromic anemia with



a blood picture resembling that of pernicious anemia and died in about two months. Two other dogs had a hyperchromic anemia which underwent spontaneous remission. Seyderhelm reported that megaloblasts were frequent in the peripheral blood and in the marrow. He considered the anemia to be related to the presence of an abnormal bacterial flora in the intestine. Lombardi,<sup>9</sup> in similar experiments, found a hypochromic anemia with poikilocytosis and anisocytosis but no megaloblasts. Another approach was made by Tönnis and his collaborators<sup>17-20</sup> who formed intestinal culs-de-sac 40 to 50 cm. long. These led to stagnation of the intestinal contents comparable with that seen in Seyderhelm's experiments. In fifteen dogs so treated, a moderate or severe anemia was found after two to four months, the color index was usually raised. This anemia could be relieved by the excision of the cul-de-sac, by administering intestinal antiseptics and by the parenteral injection of liver extract. Tönnis did not find megaloblasts in the blood. He reported that placing the cul-de-sac in the ileum was much less effective than in the jejunum. Recently, Renshaw et al.,<sup>21</sup> in an experimental study of gastrocolic fistula, reported that dogs in which such a fistula had been made developed a microcytic anemia in from two to six months, and in 2 of 7 animals this anemia later became macrocytic and hyperchromic. In these dogs, the greater part of the food passed into the small intestine, so that the anemia and other symptoms did not simply result from starvation, it was thought that contamination of the intestinal contents by regurgitation from the colon was responsible.

With the development of Castle's theory, experiments on the lines of those carried out by Seyderhelm and Tönnis were neglected in favor of resections of various parts of the gastrointestinal tract, despite the fact that the methods of these two authors produced an anemia more closely resembling pernicious anemia than those following resections. In view of the clinical similarity of pernicious anemia to the anemia often seen in association with human cases of stricture and of anastomosis of the small intestine, we have attempted to induce a similar anemia in rats by artificially producing strictures or anastomoses in their small intestines.<sup>22</sup>

The precise mechanism by which a macrocytic anemia may arise from the presence of intestinal stricture or anastomosis is not clear, but in the reported cases in man and the relevant experiments in animals one of two conditions has usually been present. The first is stagnation of the bowel content. This may occur above a stricture as in Seyderhelm's experiments. It may also occur in shortcircuited or blind loops of intestine, as are seen in some of the clinical reports of anastomotic anemia and in the experiments reported by Tönnis. The second condition is steatorrhea which may be found in the presence of gastrocolic or high jejunoileocolic fistulae. However, macrocytic anemia of intestinal origin is not invariably associated with steatorrhea<sup>9</sup> and consequently the experimental formation of gastrocolic fistulae seemed a less desirable approach to the problem than the formation of blind intestinal loops. We have, therefore, experimented with two different types of operation, intestinal stenosis and the formation of blind loops.

Adult albino rats of the Wistar strain, aged 4 to 6 months and of both sexes, were used for these experiments. The rats were kept on a diet which we have found to permit normal growth and reproduction.<sup>23</sup> Control rats kept on this diet for over

a year have maintained optimum blood counts. The normal values for blood counts in these rats will be published separately. The rat was chosen as the experimental animal because it is easy to handle and large numbers may be conveniently used.

### *Intestinal Stenosis*

In a small animal like the rat it seemed easier to create an intestinal stricture than to make an anastomosis. As the experiments described above and the records of comparable human cases did not suggest that anemia would be more likely to result from one procedure than the other, the formation of intestinal strictures was undertaken first. In all cases the stricture was made just above the cecum. The operative procedures used were partial occlusion of the small intestine with silk thread, bands of cellophane, or strips of aponeurosis, the injection of phenol or

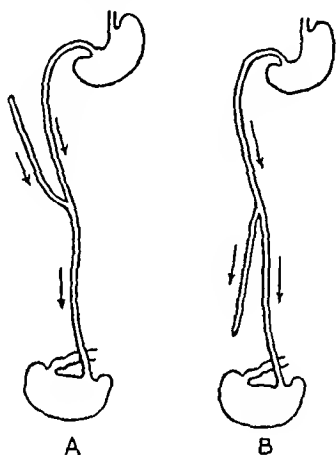


FIG 1—OPERATIONS A AND B SHOWING DIRECTION OF PERISTALSIS IN LOOPS

sodium morrhuate into the intestinal wall, the insertion into the lumen of the intestine of small perspex bobbins with only a fine channel to permit passage of the intestinal contents, and partial occlusion by sewing a linear fold in the intestine. None of these methods could be relied upon to cause obstruction of a suitable degree. In a few rats, chronic obstruction and a macrocytic anemia did develop, but as the method was so unreliable, these experiments were abandoned and attempts to form intestinal anastomoses were made.

### *Formation of Blind Intestinal Loops*

It is probable that at least a third of the small intestine can be excised without serious effect on nutrition<sup>19</sup> and in our initial experiments the loop was made that length, about 12 inches. Two types of operation were used. In operation A the direction of peristalsis was such as to tend to empty the loop (fig 1-A) and in operation B peristalsis tended to fill the loop (fig 1-B).

*Operation A* The small intestine is transected at the junction of its upper and

middle thirds. The upper end of the middle third is tied off. The lower end of the upper third is brought down to the junction of the middle and lower thirds and an end-to-side anastomosis is performed. The middle third of the small intestine is thus converted into a self-emptying blind loop. Following this operation, macrocytic anemia was observed in a small porportion of animals (table 2). It was found that anemia developed only when there was some stenosis at the site of the anastomosis leading to dilatation and filling of part of the loop. The number of anemic animals obtained was small compared to the effort expended and the yield tended to decrease as our technical skill improved. This operation was accordingly abandoned in favor of operation B.

*Operation B.* In the first form of this operation the small intestine was transected at the junction of the middle and lower thirds. The lower end of the middle third

TABLE 2.—Frequency of Anemia with Self Emptying Loops

	Number of rats	Per cent of total
Operative mortality	73	38
Macrocytic anemia	21	11
Normo or microcytic anemia	11	6
Retained normal blood count	86	45
Total	191	100

TABLE 3.—The Effect of Various Lengths of Self Filling Blind Loops

Length of Loop	Number of rats	Survived 3 wks. or more	Macrocytic anemia
12 inches	26	7	7
6 inches	12	4	1
3 inches	24	17	13

is tied off, and the upper end of the lower third of the small intestine is brought up to the junction of the upper and middle thirds, where an end-to-side anastomosis is performed. This operation is technically more difficult than operation A. The waves of peristalsis tend to break down the anastomosis, which must be much more firmly secured than in operation A. A remarkable dilatation of the blind loop occurs. With an effective self-filling loop all the animals die prematurely, some with and some without anemia. The results following this operation were encouraging and the technic was adopted as the basis for subsequent experiments to determine the optimum site and length of the blind loop (tables 3 and 4). It will be seen (table 3) that a twelve inch loop gave a very high percentage yield of anemic rats, but these animals did not survive more than four to six weeks and could not be saved by liver therapy. The results with 6 inch loops were not much better. With a 3 inch loop, however, the operative mortality was not excessive, the anemia developed more gradually, usually eight to ten weeks after operation, and the rats could generally be saved by liver treatment.

It seems clear (table 4) that it is important to place the blind loop in the middle or upper part of the small intestine. With low ileal loops the yield of anemic rats was very small. The results with jejunal and mid-intestinal loops were comparable but the latter were somewhat easier to make. The formation of a 3 inch self-filling blind loop about the middle of the small intestine has been consequently adopted as a standard technic for our subsequent experiments, and the operation will be described in detail.

#### *Technic of Standard Operation*

An intraperitoneal injection of 30 mgm of pentobarbitone in 0.1 ml of sterile distilled water is administered, and the anesthetic is completed and maintained

TABLE 4.—*The Effect of Various Positions of a Three Inch Self Filling Blind Loop*

Position of Loop	Number of rats	Survived 3 wks or more	Macrocytic anemia
Jejunum	18	10	5
Mid intestine	18	13	7
Just above caecum	18	12	1

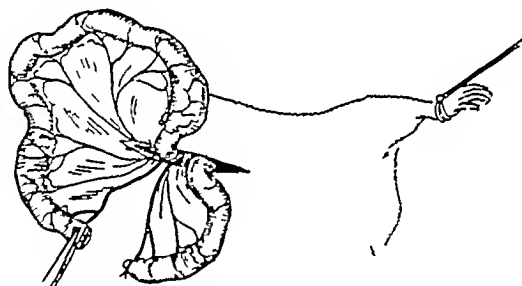


FIG. 2.—TRANSECTION OF SMALL INTESTINE

with ether. The hair on the abdomen is clipped short. The animal is tied on its back and the skin over the abdomen is cleansed with ether and painted with an aqueous solution of flavine. Observing aseptic technic, the abdominal cavity is opened through a 4 cm. mid-line incision. The cecum is identified and brought out. Working upwards from the cecum, approximately 40 cm. of small intestine is gently withdrawn. The upper end of the segment is clamped and divided. The free end above the point of division is securely tied off with a fine ligature (fig. 2). The free end below the point of division is retained outside while the remaining portion of the lower segment and the cecum are returned to the abdominal cavity. Working upwards from the point of division, another 7 or 8 cm. (3 inch) length of small intestine is carefully withdrawn. A loop marking the upper end of this portion is retained outside and the remainder of the segment is returned to the abdominal cavity. An opening in the loop about 2 mm. in diameter is created by making a small transverse incision through its antimesenteric surface. The open

upper end of lower segment is approximated to the opening in the loop (fig 3) and an end-to-side anastomosis is performed. Accurate apposition of the open ends is secured with four stay sutures and the anastomosis is completed by over sewing serosa to serosa (fig 4). The middle 8 cm. of the small intestine is thus converted into a self-filling blind loop. The peritoneum and muscles are closed

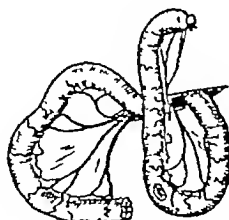


FIG 3 — LOOP FASHIONED OPEN ENDS IN APPPOSITION

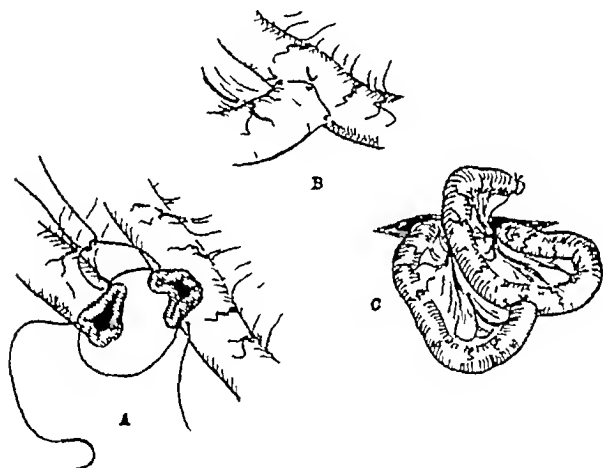


FIG 4 — (A) — Placing the stay sutures (B) — Stay sutures completed (C) — Anastomosis complete

with a single continuous suture. The skin is closed with a continuous eversion suture. No dressing is applied.

#### *Description of the Anemia*

Macrocytic anemia has developed in a proportion of the animals subjected to each of the operations we have used and its characteristics have been the same irrespective of the type of operation. A hypochromic normocytic anemia was occasionally observed following operation A<sup>12</sup> but it has not been encountered in the later experiments.

Of the first 148 rats subjected to the standard operation, 104 (70 per cent) survived its immediate effects and of these 42 (44 per cent) developed macrocytic

anemia after an average interval of 74 postoperative days. The length of the interval ranged from 26 days to 158 days. As the later animals in this group have so far been observed for only three months, it is probable that more of them will eventually become anemic. Table 5 gives representative examples, at various levels, of the blood findings in ten consecutive anemic animals of this group. A further large number of animals is at present under observation.

Once anemia has appeared following the standard operation, it is progressive and appears to be almost invariably fatal within a month if untreated. Of 12 such untreated animals only 3 survived for more than eight days and these 3 were all dead by the thirty-fourth day. Anemic animals tend to lose weight but they do not show any external evidence of deficiency disease.

The normal hemoglobin level in our rats is about 14 Gm. per 100 ml. and anemia has not been diagnosed unless the hemoglobin level has fallen below 10 Gm. per

TABLE 5—The Blood Picture of Rats with Macrocytic Anemia

Rat	Hemo- globin (Gm. per 100 ml.)	Eryth- rocytes (mil- lions)	Reticu- lo- cytes %	Hemat- ocrit %	M.C.H. ( $\mu$ g.)	M.C.V. (c. $\mu$ )	$\bar{V}$ M.C.H.C.	M.C.D. ( $\mu$ )	Leuko- cytes (thou- sands)	Plate- lets (thou- sands)
Normal Mean	13.8	8.7	4.0	42	16	49	34	6.14	20	531
1 13A	5.8	3.3	2.7	22.2	18	67	38	6.44	44	603
2 13F	3.2	2.6	5.2	13.6	13	52	43	6.42	16	745
3 14D	8.6	4.1	1.8	22.7	21	55	27	6.50	15	376
4 14E	7.6	5.0	.9	25	15	50	33	6.50	17	361
5 15A	7.6	5.7	2.8	28.5	13	50	37	6.55	18	955
6 15E	7.0	4.8	2.1	24.5	15	51	35	6.61	28	895
7 15F	4.1	3.0	7.1	18.6	14	62	45	7.33	21	330
8 16C	8.3	5.0	2.3	28.6	17	57	34	6.44	25	797
9 16E	4.9	3.4	4.0	17.8	15	53	36	6.37	15	390
10 36A	8.9	5.8	1.1	30	15	52	34	7.01	23	—

100 ml. Films of tail blood from the anemic animals were prepared in the usual way and stained with May-Grünwald-Giemsa. Staining of the erythrocytes varied considerably. In most fields they displayed central pallor but in a few fields staining was more uniform. Target cells were occasionally seen. Macrocytosis, anisocytosis and diffuse polychromasia were well marked in all instances. A slight degree of poikilocytosis and punctate basophilia was usually present. Nucleated red blood cells, chiefly polychromatic normoblasts, were frequently seen. No megaloblasts were observed. The mean erythrocyte diameter of each of the anemic animals in table 5 was estimated by the method of Price-Jones.<sup>20a</sup> In every case the Price-Jones curve exceeded the ideal  $+3\sigma$  curve. The chances are less than 1 in 770 that such a curve could be normal. These findings clearly demonstrated that the anemia was macrocytic. The hemoglobin readings ranged from 9.9 Gm. per 100 ml. to 2.7 Gm. per 100 ml. The erythrocyte counts ranged from 6.9 millions per cu. mm. to 2.4 millions per cu. mm. and were usually under 6 million per cu. mm. Reticulocyte counts were elevated and showed an exaggeration of the wide range found in nor-

mal animals Platelet counts and leukocyte counts were all within the normal range as were the differential white cell counts The corpuscular constants also fell within the normal range The wide variability of the corpuscular constants in the rat renders them of little value in the study of this experimental anemia

Bone-marrow smears were made from normal and anemic rats by a method which did not involve sacrifice of the animal <sup>8</sup> Smears from rats with macrocytic anemia showed normal or increased cellularity, the most striking change in distribution was an increase in the proportion of earlier red-cell precursors In smears from normal rats the predominant cells of the erythroblast series are small polychromatic normoblasts with dense or cartwheel nuclei this distribution was unaltered in rats subjected to repeated hemorrhage, but in rats with macrocytic anemia there were increased numbers of proerythroblasts and basophil erythroblasts The degree of this change varied considerably in different rats No cells were seen which corresponded exactly with the megaloblasts of human pernicious anemia, but there were some cells of the erythrocyte series which appeared abnormal in that the nucleus did not have the compact character seen in cells from normal marrow, and occasional cells had a closer resemblance to the megaloblast Marrow from anemic rats showed increased numbers of plasma cells, but there were no appreciable changes in the myeloid series

The rat is a small animal with a brisk metabolism and does not tolerate anemia well If the hemoglobin level falls much below 7 Gm per 100 ml the rat becomes torpid, loses weight and its resistance to infection becomes impaired In such circumstances, the state of ill health may become irreversible Consequently, therapeutic tests are probably best made at a level of 7-9 Gm of hemoglobin per 100 ml Preliminary observations have suggested that this experimental macrocytic anemia responds to treatment with liver extract and with pteroylglutamic acid <sup>12</sup> Experiments are now in progress to determine the effects of liver extract, pteroylglutamic acid and vitamin B<sub>12</sub> and the results will be reported separately

#### DISCUSSION

These experiments have shown that macrocytic anemia can be produced in the rat by the formation of a blind loop in the small intestine They have also confirmed two observations made by Tönnis and his collaborators in similar operations on dogs The first is that the loop must be designed so that it is filled by the action of peristalsis The second is that it must be placed in the upper or middle thirds of the small intestine The implication is that the loop must be filled with stagnant undigested intestinal contents if it is to have a pathologic effect Tönnis went on to infer that the pathologic changes are produced by the absorption of toxic substances from the loop He noted that his dogs died in a shorter time on a meat diet than on a lacto-vegetarian diet, and he observed toxic changes in the liver and the kidneys

The livers in our rats have shown no microscopic abnormality Moreover, it is characteristic that the rats appear well for two or three months after the operation and then quite suddenly become anemic and ill This suggests that the decisive event may be a change in the flora of the loop and, possibly, in the remainder

of the small intestine. This may lead to the loss of an organism which synthesizes hemopoietic material, or the predominance of an organism which uses up hemopoietic material, or the presence of an organism which produces an antagonist to hemopoiesis. Such an antagonist might interfere with the Castle reaction, as the fish tapeworm does,<sup>41</sup> or with the action of vitamin B<sub>12</sub> or folic acid. We have some evidence that life can be prolonged by treatment of the rats with Anahaemin, folic acid or vitamin B<sub>12</sub>. However, the experimental conditions are probably complex and it would be a mistake to discuss them solely in terms of anemia. A number of our rats have died without anemia and if, as we imagine, the blind loop leads to a conditioned deficiency, it is almost certainly a deficiency of more than one substance. The chief value of the blind loop preparation may be that it affords a relatively simple method of disturbing the equilibrium in the small intestine and studying the consequent changes in absorption and nutrition.

It is interesting that it is possible to produce a macrocytic anemia in animals by operations on the small intestine which lead to stagnation of the intestinal contents, whereas operations on the stomach consistently fail. In the human subject surgical procedures which involve the intestine occasionally lead to an anemia which closely resembles pernicious anemia and which responds to liver or folic acid, whereas removal of the stomach is rarely followed by pernicious anemia. Some of the features of true Addisonian anemia are still unexplained—the remissions and relapses, and the small proportion of patients with achlorhydria who develop the disease. These phenomena have been attributed to variations in the secretion of Castle's intrinsic factor, but the possibility that they are due to variations in the flora or function of the small intestine cannot be excluded.

#### SUMMARY

1. The literature concerning attempts to produce macrocytic anemia of the liver-deficiency type in animals by operations on the gastrointestinal tract has been reviewed. Operations on the stomach have failed consistently to produce such an anemia, but success has been achieved by operations on the small intestine with the creation of blind loops or intestinal stenosis.

2. The technic we have used to produce macrocytic anemia in the rat is described in detail. The essentials are that the blind loop should fill with peristalsis and that it should not be too low down in the small intestine.

3. Anemia does not usually develop until an interval of several weeks or months after the operation. It is then macrocytic in type and acute in course.

4. The anemia is probably dependent on stagnation in the blind loop and a change in the bacterial flora of the small intestine.

#### REFERENCES

1. ARON, E. AND BAUER, R. Anémie expérimentale chez le chien. *Compt. rend. Soc. de biol.* 113: 1065, 1933.
2. BACHRACH, W. H. AND FOEELSON, S. J. The role of the upper gastro-intestinal tract in the etiology of pernicious anaemia. *J. Lab. & Clin. Med.* 24: 249, 1938.
3. BARKER, W. H. AND HUMMEL, L. E. Macrocytic anemia in association with intestinal strictures and anastomoses. *Bull. Johns Hopkins Hosp.* 64: 215, 1939.



- <sup>1</sup> BENCE J Die Rolle des Magens und der Leber in der Pathologie der perniziösen Anämie *Ztschr f klin Med* 126 127 1933
- <sup>2</sup> — Die Beziehungen der experimentellen agastrischen Anämie zur Perniciosa *Ztschr f klin Med* 130 275 1936
- <sup>3</sup> BROWN M R Deficiency syndromes secondary to short circuited small intestine *J Clin Investga tion* 17 529 1948
- <sup>4</sup> BUSSABAROER R A CUTHBERT F P AND IVY, A C Studies on the anemia of pregnancy in gastrectomized and normal dogs *J Lab & Clin Med* 24 24 1938
- <sup>5</sup> CAMERON D G AND WATSON G M Femoral bone marrow biopsy in the albino rat *Blood* 3 291, 1948
- <sup>6</sup> — — AND WITTS L J The clinical association of macrocytic anemia with intestinal stricture and anastomosis *Blood* this issue p 793
- <sup>7</sup> DEXTER S O HEINLE R W FOX H J AND CASTLE W B Basis of hematopoietic activity in pernicious anemia of desiccated hog ileum *J Clin Investigation* 18 473 1939
- <sup>8</sup> FOX H J AND CASTLE W B Observations on etiologic relationship of achylia gastrica to pernicious anemia difference in site of secretion of intrinsic factor in hog and in human stomach *Am J M. S.* 203 18 1942
- <sup>9</sup> GRIER A J GOOMAN L S AND CLAIBORN L N Studies in pernicious anemia Extirpation of intrinsic factor sources 16 666 1937
- <sup>10</sup> — — AND CLAIBORN L N Studies in Pernicious Anemia Surgical elimination of Gastro-Intestinal Factors *J Clin Invest* 15 474 1936
- <sup>11</sup> HAUSWIATH L, AND SILBERSTEIN F Experimentelle Beiträge zur Physiologie und Pathologie des Duodenums *Med Klin* 24 1795 1928
- <sup>12</sup> HIGGINS G M Effect of synthetic lactobacillus casei factor on the blood changes induced by gastrectomy in the rat Abstracts XVII International Physiological Congress Oxford University Press 22, 1947
- <sup>13</sup> HORSTER H Experimentelle Therapie bei intestinaler Autointoxication *Ztschr f d ges exper Med* 95 514 1935
- <sup>14</sup> IVY A C MORGAN J E AND FARRELL J I The effects of total gastrectomy *Surg Gynec & Obst* 53 611, 1931
- <sup>15</sup> JENSENIUS H Results of Experimental Resections of the Small Intestine on Dogs Copenhagen and London, 1945
- <sup>16</sup> LOMBARDI E. Solla etiologia del l anemia perniciosa *Riforma med* 44 98 1928
- <sup>17</sup> MALMROS R (Diskussion Dansk Selskab for intern Medicin d 20/5 1938) *Ugesk f Læger* 111 734 1939
- <sup>18</sup> MANN F C AND HAWAMURA K (1922) Duodenectomy (an experimental Study) *Ann Surg* 75 208
- <sup>19</sup> MEULENORACHT E The presence of the antianaemic factor in preparations of dried stomach substance from the cardia fundus and pylorus respectively *Acta med Scandinav* 82 352 1934
- <sup>20</sup> — Continued investigations on the presence of the antianaemic factor in preparations of dried stomach substance from the cardia fundus and pylorus and the duodenum V *Acta med Scandinav* 85 79 1935
- <sup>21</sup> MILLER D K AND RHOADS C P The experimental production of loss of hematopoietic elements of the gastric secretion and of the liver in swine with achlorhydria and anemia *J Clin Investigation* 14 153 1935
- <sup>22</sup> PETAI, S AND JENSENIUS H Experimental studies on production of pernicious anemia by operation on the digestive tract I *Acta med Scandinav* 106 274 1941
- <sup>23</sup> — — NORGAARD F AND THYSSON E Results of extensive resection of proximal part of small intestine (on pups) *Acta Med Scandinav* 111 116 1942
- <sup>24</sup> — — MØRGAARD F AND JENSENIUS H Experimental studies on production of pernicious anemia by operation on the digestive tract II *Acta med Scandinav* 107 506 1941
- <sup>25</sup> — — JENSENIUS H AND THYSSON E Experimental studies on production of pernicious anemia by operation on the digestive tract III *Acta med Scandinav* 107 532 1941
- <sup>26</sup> — — MØRGAARD F AND THYSSON E Experimental studies on production of pernicious anemia by operation on the digestive tract IV *Acta med Scandinav* 111 75 1942

- <sup>71a</sup> PRICE JONES C Red Blood Cell Diameters London, 1933
- <sup>71</sup> RENSCHAW R J F, TEMPLETON F E AND KISBADDEN R M Gastrocolic fistula A clinical and experimental study *Gastroenterology* 7 511 1946
- <sup>72</sup> SCHEMENSKY W Zur Pathologie der perniziösen Anämie Therapeutische Erfolge mit Verfütterung getrockneten Schweinedickdärmpulvers *Ztschr f klin Med* 128 428 1935
- <sup>73</sup> SEYDERHELM R LEHMANN W AND WICHELS P Experimentelle intestinale perniziöse Anämie beim Hund *Klin Wchnschr* 2 1439 1924
- <sup>74</sup> — — AND — Intestinale perniziöse Anämie beim Hund durch experimentelle Dünndarmstricture *Krankheitsforschung* 4 263 1927
- <sup>75</sup> SHARPE E A MCKEAN R M AND VONDER HEIDE E C Pernicious anemia Behaviour of various extracts of stomach and duodenum used to induce remissions *Ann Int Med* 4 1282 1931
- <sup>76</sup> SILBERSTEIN F AND HAUSWIRTH L Experimentelle Untersuchungen über die Bedeutung des Duodenums für den Stoffwechsel *Schweiz med Wchnschr* 61 885 1931
- <sup>77</sup> STASSOFF B Experimentelle Untersuchungen über die kompensatorischen Vorgänge bei Darmresektionen *Beitr z klin Chir* 29 527 1914
- <sup>78</sup> TÖNNIS W AND BRUSIS A Veränderungen des morphologischen Blutbildes bei akuter und chronischer Darminhaltstauung *Deutsche Ztschr f Chir* 233 133 1931
- <sup>79</sup> — HORSTER H REIMERS C AND RÜDEL C Experimentelle Untersuchungen zur intestinalen Autointoxikation I *Ztschr f d ges exper Med* 84 728 1932
- <sup>80</sup> UOTILA U On the presence of arianaemic activity in the ileal part of the small intestine *Acta med Scandinav* 89 50 1936
- <sup>81</sup> VON BONSDOERFF B Pernicious anaemia caused by *Diphyllobothrium latum* in the light of recent investigations *Blood* 3 91 1948
- <sup>82</sup> WATSON G M CAMERON D G AND WITTS L J Experimental macrocytic anaemia in the rat *Lancet* 2 404 1948
- <sup>83</sup> WINTROBE M M SAMTER M AND LIKO, H Morphological changes in the blood of pigs associated with deficiency of water-soluble vitamins and other substances contained in yeast *Bull Johns Hopkins Hosp* 64 399 1939

## THE BLOOD COUNTS OF THE ADULT ALBINO RAT

By D. G. CAMERON, B.Sc., M.D., AND G. M. WATSON, D.Phil., M.R.C.P.

IN THE course of the investigation of an experimental anemia of adult albino rats, it was necessary to determine the normal range of values for the blood counts of these animals. Normal values for rat blood counts have been reported by several authors<sup>1-9</sup> but as the published figures show discrepancies and are incomplete in some respects, our findings may be of general interest.

### METHODS

The animals used in these experiments were adult albino rats of the Wistar strain, of both sexes and aged 4 months or older. They were maintained on a synthetic diet of casein, corn starch, cod liver and arachis oils with mineral and vitamin supplements. We have found<sup>10</sup> that this diet permits normal growth and reproduction. Animals kept on it for periods of twelve months have maintained optimum blood counts.

Free flowing blood was collected from the tail after warming it in hot water and snipping a bit from the end with scissors or scalpel. The animals were not restrained and were alarmed as little as possible.

Erythrocyte and leukocyte counts were made with the usual pipets and diluting fluids but as the counts were high, it was found advisable to draw blood only to the 0.3 mark in the pipets. Hemoglobin was estimated with a previously calibrated Evelyn photoelectric colorimeter using 20 cu. mm. of blood diluted with 6 ml. of 0.4 per cent ammonia. Reticulocytes were counted by a wet film method. Blood was mixed with the diluting fluid of citrated normal saline and brilliant cresyl blue in a leukocyte pipet. A drop of the mixed blood and stain was placed on a glass slide covered and sealed with liquid paraffin. Platelets were counted in the same preparation and their absolute numbers were calculated from the erythrocyte count. For hematocrit readings a heparinized 1 mm. bore tube closed at one end and having a cup-shaped expansion at the other was used. A few drops of blood were collected in the cup, the tube was agitated to ensure mixing and then centrifuged for thirty minutes at 5000 r.p.m. The readings were made by laying the tube on squared paper. Mean corpuscular diameters were determined by Price Jones<sup>11</sup> method. An eosin-stained blood film was projected at 2000 diameters using a dry optical system and the average diameters of 500 erythrocytes were measured. The mean diameter and standard deviation were calculated for the erythrocytes of each animal.

### RESULTS

The results for male and female rats are presented separately in tables 1 and 2. Table 3 shows the range of values obtained in differential leukocyte counts.

### COMMENTS

The published figures mentioned above do not show much variation in the values found for hemoglobin content, which are in the range 13 to 15 grams per cent. The erythrocyte counts, however, vary from 6.60 million<sup>7</sup> to 9.53 million<sup>8</sup> per cu. mm. Our figures lie between these extremes. Our values for the normal leukocyte count, as well as those of Thewlis and Meyer<sup>9</sup> and Quimby et al.,<sup>12</sup> are much higher than the figures usually reported. The reason for this discrepancy is not apparent. It is probable that strain differences and variations in diet are partly responsible. Farris<sup>13</sup> reported a lymphocytosis under emotional stimulus, but

From the Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Oxford, England.

found no increase in the total leukocyte count. Recently, Quimby et al.<sup>13</sup> have shown that the leukocyte count of heart blood is much lower than that of tail blood.

TABLE 1.—*Blood Counts of the Adult Male Albino Rat*

	Number of observations	Mean	Standard error of the mean
Erythrocytes (millions per cu mm)	71	8.50	0.106
Reticulocytes (% of erythrocytes)	69	4.5	0.351
Hemoglobin (grams per 100 ml)	71	14.6	0.191
Hematocrit (% packed red cells)	69	43.5	0.642
M.C.V. (cubic microns)	69	52.2	0.707
M.C.H. (micromicrograms)	69	17.2	0.218
M.C.H.C. (per cent)	69	30.0	0.327
M.C.D. (microns)	15	5.98	0.061
Standard deviation of red cell diameters	15	0.46	0.023
Leukocytes (thousands per cu mm)	69	21.4	0.727
Platelets (thousands per cu mm)	59	673.0	40.9

TABLE 2.—*Blood Counts of the Adult Female Albino Rat*

	Number of observations	Mean	Standard error of the mean
Erythrocytes (millions per cu mm)	200	8.70	0.069
Reticulocytes (% of erythrocytes)	200	4.0	0.127
Hemoglobin (grams per 100 ml)	200	13.8	0.068
Hematocrit (% packed red cells)	76	41.8	0.409
M.C.V. (cubic microns)	76	49.0	0.600
M.C.H. (micromicrograms)	76	16.0	0.191
M.C.H.C. (per cent)	76	34.0	0.323
M.C.D. (microns)	20	6.14	0.032
Standard deviation of red cell diameters	20	0.42	0.018
Leukocytes (thousands per cu mm)	200	20.4	0.582
Platelets (thousands per cu mm.)	15	531.8	15.16

TABLE 3.—*Differential Leukocyte Counts of the Adult Albino Rat*

Cell Type	Average %	Range %
Neutrophils	15	8-24
Eosinophils	1	0-4
Lymphocytes	81	70-89
Monocytes	3	1-6

Note.—22 ♂ and 22 ♀ animals. No sex difference was noticed in the differential counts.

We have found small differences between the sexes in hemoglobin values, erythrocyte counts and hematocrit readings. The divergence is statistically significant for the hemoglobin values, but in the other cases probably does not represent a real difference due to sex.

## SUMMARY

The normal values for blood counts in the adult albino rat are presented

## REFERENCES

- <sup>1</sup> DONALDSON, H. H. *The Rat*. Memoirs of the Wistar Institute, 1924.
- <sup>2</sup> SCARBOROUGH, R. A. The blood picture of normal laboratory animals. *Yale J. Biol. Med.* 3: 547-4. 69, 1931.
- <sup>3</sup> WILLS, L., AND MEHTA, M. M. Determination of normal blood standards for the Nutritional Laboratory's stock albino rat. *Ind. J. M. Research* 18: 307, 1930-31.
- <sup>4</sup> ORTEN, J. M., AND SMITH, A. H. The proportion of reticulocytes in the blood of albino rats. *Am. J. Physiol.* 108: 66, 1934.
- <sup>5</sup> DRABKIN, D. L., AND FITZHUOH, T. JR. A comparison of the normal blood picture of rats (at various ages) of two different colonies reared upon different stock rations. *Am. J. Physiol.* 108: 61, 1934.
- <sup>6</sup> ENZMANN, E. V. The changes in hemoglobin concentration of blood of growing rats. *Am. J. Physiol.* 108: 373, 1934.
- <sup>7</sup> WINTROBE, M. M., SCHUMACKER, H. B. JR., AND SCHMIDT, W. J. Values for number size and hemoglobin content of erythrocytes in normal dogs, rabbits and rats. *Am. J. Physiol.* 114: 502, 1936.
- <sup>8</sup> CRESKOFF, A. J., FITZHUOH, T., AND FARRIS, E. J. In: GRIFFITH AND FARRIS. *The Rat in Laboratory Investigation*. Philadelphia, 1942. Pp. 351-359.
- <sup>9</sup> THEWLIS, E. W., AND MEYER, O. O. The blood count of normal white rats. *Anat. Rec.* 82: 115, 1942.
- <sup>10</sup> CAMERON, D. G., AND WATSON, G. M. Femoral bone marrow biopsy in the albino rat. *Blood* 3: 292, 1948.
- <sup>11</sup> PRICE JONES, C. *Red Blood Cell Diameters*. London, 1933.
- <sup>12</sup> FARRIS, E. J. Emotional lymphocytosis in the albino rat. *Am. J. Anat.* 63: 325, 1938.
- <sup>13</sup> QUIMBY, F. H., SAXON, P. A., AND GOFF, L. G. Total white cell counts of peripheral and heart blood of the rat. *Science* 107: 447, 1948.

# OBSERVATIONS ON THE EFFECT OF AN ANIMAL PROTEIN FACTOR CONCENTRATE ON PERSONS WITH THE MACROCYTIC ANEMIA OF PERNICIOUS ANEMIA, OF NUTRITIONAL MACROCYTIC ANEMIA AND OF SPRUE, AND ON PERSONS WITH NUTRITIONAL GLOSSITIS

By TOM D SPIES M D , GUILLERMO GARCIA LOPEZ, M D , FERNANDO MILANES, M D , ROBERT E STONL, M D , RUBEN LOPEZ TOCA, M D , TOMAS ARAMBURU, M D , AND SAM KARTUS, M D

FOR THE past fifteen years, investigators have been working intensively on an illusive vitamin, or a complex of closely related factors, found in association with proteins of animal origin. For a number of years there has been evidence that soya bean meal was not adequate as the only source of protein in poultry feeds.<sup>1</sup> The hatchability of eggs produced by hens fed these diets was low, whereas this defect could be remedied by feeding meat scraps.

Later it was found that a supplement of dried cow manure was effective in increasing the egg production and the hatchability of the eggs from hens fed on such a restricted diet.<sup>2</sup> Whitson, Hammond, Titus, and Bird<sup>3</sup> concluded that the substance was not a protein or any known vitamin. Bird, Rubin, Whitson, and Haynes<sup>4</sup> aided in the search for the hatchability factor by investigating a large number of widely used foodstuffs, and Mishler, Carrick, and Hauge<sup>5</sup> found that the addition of fish solubles supplied a missing factor or factors not present in a vegetable protein ration supplemented with vitamins. Rubin, Bird, and Rothchild<sup>6</sup> demonstrated the presence of a chick growth factor in hen feces.

As these studies progressed on the new factor or factors necessary for the hatchability, growth, and viability in chicks, Ross, Phillips, and Bohstedt,<sup>7</sup> Cunha,<sup>8</sup> Spitzer and Phillips,<sup>9</sup> and Cary, Hartman, Dryden, and Likely<sup>10</sup> independently showed that proper growth, reproduction, and lactation did not occur in rats fed a diet of highly purified casein as the source of their protein and that they required an unidentified factor.

More recently, Zucker, Zucker, Babcock, and Hollister<sup>11</sup> fed rats a purified diet containing protein and all of the known essential vitamins. The female rats maintained on this diet reproduced normally and had normal lactation, but the young born of such females frequently died soon after weaning. Crude casein, fish solubles, or liver extract corrected the deficiency, and the new factor was tentatively named zoopherin. The authors pointed out that it is not the same as the fat-soluble animal protein factor of Heuser, Norris, Lucas, and Combs<sup>12</sup> and of Johnson, Carrick, and Roberts.<sup>13</sup>

Northwestern University Studies in Nutrition at the Hillman Hospital Birmingham Alabama and at the General Calixto Garcia Hospital Havana Cuba in cooperation with the University of Havana From the Department of Nutrition and Metabolism Northwestern University

This study was supported by grants from the Birmingham Citizens Committee and from Lederle Laboratories Inc

The animal protein factor concentrate was produced by micro-organisms and was supplied by Dr T H Jukes of Lederle Laboratories Inc

In another field of investigation, workers found a long-sought-for antipernicious anemia factor of liver. This substance was isolated independently by workers in the United States<sup>14</sup> and in England.<sup>15</sup> Ott, Rickes, and Wood<sup>16</sup> have reported that it possesses animal protein factor activity for the chick and concluded that this vitamin, termed B<sub>12</sub>, is identical or closely related to the animal protein factor from other sources.

The chemical nature and structure of the new vitamin B<sub>12</sub> is not fully known. Its molecular weight is between 1,350 and 1,750. It is a cobalt complex and contains phosphorus. Irrespective of whether this material should prove to be identical with one or more of the various chick growth and hatchability factors, vitamin B<sub>12</sub> is of great interest in human nutrition. There are substantial differences in nutrient needs among the species, but we must suppose that man, as well as other animals, is greatly dependent on such factors in his diet for protection. The appraisal of the worth of these particular substances to human beings is being determined by observations of therapeutic responses and by biochemical examinations of the body tissues and fluids.

It has been shown recently that concentrates from a micro-organism having animal protein factor activity were effective in inducing blood regeneration in two patients with pernicious anemia.<sup>17</sup>

The studies reported in this communication were devised to provide the answers to the following questions:

1. Could we confirm the findings of Stokstad and his associates that a concentrate produced by micro-organisms, which acts as a source of animal protein factor as measured by assay with chicks, would be effective in producing a positive hemopoietic response in persons with Addisonian pernicious anemia in relapse?

2. Would this same concentrate be effective in producing blood regeneration in persons with nutritional macrocytic anemia in relapse?

3. Would this concentrate produce a blood response in persons with the macrocytic anemia of tropical sprue in hemopoietic relapse?

4. Would it produce a remission of nutritional glossitis unassociated with macrocytic anemia?

For these studies we selected 5 cases of pernicious anemia, 4 cases of nutritional macrocytic anemia, and 3 cases of nutritional glossitis from the Nutrition Clinic of the Hillman Hospital, Birmingham, Alabama, and 3 cases of tropical sprue from the Pabellon Especial, the ward for the study of sprue, in the General Calixto Garcia Hospital, Havana, Cuba.

An important criterion in the selection of all the patients was a painful, fiery red tongue.

The following four criteria were used in the selection of all the patients with pernicious anemia, nutritional macrocytic anemia, and tropical sprue: (1) macrocytic hyperchromic anemia, (2) red blood cell count of 2,500,000 or less, (3) color index of 1.0 or more, (4) megaloblastic arrest of the sternal bone marrow.

An additional criterion for pernicious anemia was the absence of free hydrochloric acid in the gastric contents after histamine stimulation. Additional criteria for nutritional macrocytic anemia were the presence of free hydrochloric acid in

the gastric contents and diarrhea with liquid to soft, brown stools. Additional criteria for tropical sprue were the presence of free hydrochloric acid in the gastric contents, a flat glucose tolerance curve, acid steatorrhea, and loss in body weight.

All the patients except Case 1 were ambulatory and came to the hospital daily for observation and treatment. Repeated gastric analyses were made in each case. Daily studies of the peripheral blood included red and white blood cell counts, hemoglobin determinations, and reticulocyte counts made by methods previously described.<sup>18</sup> Bone marrow studies were made prior to therapy.

After the baseline determinations were completed, animal protein factor concentrate was injected in amounts ranging from a total of 5 cc. in a period of twenty-three days to 5 cc. daily for fourteen days. The following brief representative case histories illustrate the responses of one case of pernicious anemia, one case of nutritional macrocytic anemia, one case of tropical sprue, and one case of nutritional glossitis unassociated with anemia.

### CASE REPORTS

*Case 1.* M. D., a 62-year-old white woman, was admitted to the Hillman Hospital, Birmingham, Alabama, in October, 1948, complaining of weak spells and numbness and tingling of the hands, legs, and feet. Because her memory was poor, she was unable to give an accurate history. She stated that her illness began rather suddenly four years previously and was characterized by weakness and epigastric distress. At that time she remained in bed for three months but could not recall having had any specific treatment. She gained enough strength slowly to become ambulatory but she continued to feel weak. In 1947, after a period of progressive weakness, she had again become bedridden. She lost her appetite, lost weight, and developed edema of the ankles and numbness and tingling of the feet, legs, and hands. She was in bed for seven months and had numerous injections which she thought were liver extract. Following this treatment, she was able to be up for three months although the numbness of the feet, legs, and hands persisted and she felt weak. The following seven months she spent most of the time in bed. She failed to gain strength and came to the Nutrition Clinic seeking treatment. She was admitted to the Hillman Hospital, where physical examination showed a poorly developed, poorly nourished woman. The skin was pale and showed decreased elasticity. The conjunctivae were very pale and the sclerae had a slight icteric tint. The tongue showed mild glossitis. She walked with a staggering gait and watched the movement of her feet carefully. The calves were slightly tender to pressure. There was hypesthesia of the hands and forearms, hypesthesia of the fingers and hyperesthesia of the lower thighs and the legs. Touch perception in the feet was absent. There was slight blunting of position sense. The knee jerks were hyperactive. Vibratory sensation showed a great decrease at the ankles and a slight decrease at the knees and wrists. Repeated gastric analyses showed no free hydrochloric acid in the gastric juice after histamine stimulation. The blood values on admission were: red blood cells 1.73 million, white blood cells 1,900, hemoglobin 6.6 grams (42 per cent), and reticulocytes 1.6 per cent. The patient was given 5 cc. of animal protein factor concentrate intramuscularly each day for fourteen days. The reticulocytes began to rise on the fourth day of therapy and reached a peak of 16.4 per cent on the seventh day (fig. 1). Three weeks after therapy was initiated, the red blood cell count increased to 3.41 million, the white blood cell count increased to 8,750, the hemoglobin increased to 9.3 grams (60 per cent), and the reticulocytes decreased to 2.8 per cent. Eight weeks after therapy was discontinued, the red blood cell count was 3.88 million, the white blood cell count 7,750, the hemoglobin 12.4 grams (80 per cent), and the reticulocytes 1.0 per cent. The sixth day following the initiation of therapy, she had a definite improvement in appetite and consumed more food than she had previously. By the seventh day the signs of glossitis had subsided, and she stated that she felt stronger and began to spend time out of bed. At this time she seemed a little more alert mentally and she began to complain more severely of pains and paresthesia of all the extremities. Objectively, no improvement in the neurologic status could be detected except that she seemed to walk a little better and this might very well be attributed to a gain in strength rather than to any real improvement in the nervous system.



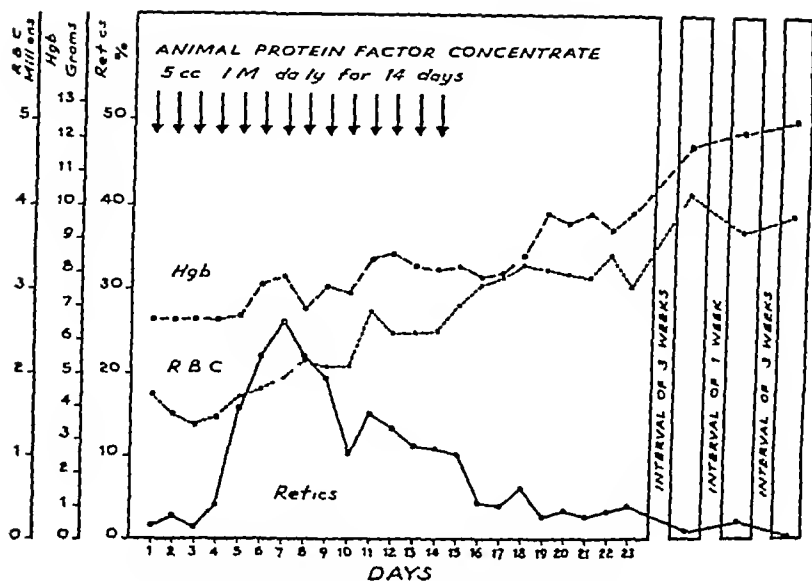


FIG 1—HEMOPOIETIC RESPONSE OF A PATIENT (M. D.) WITH PERNICIOUS ANEMIA TO ANIMAL PROTEIN FACTOR CONCENTRATE

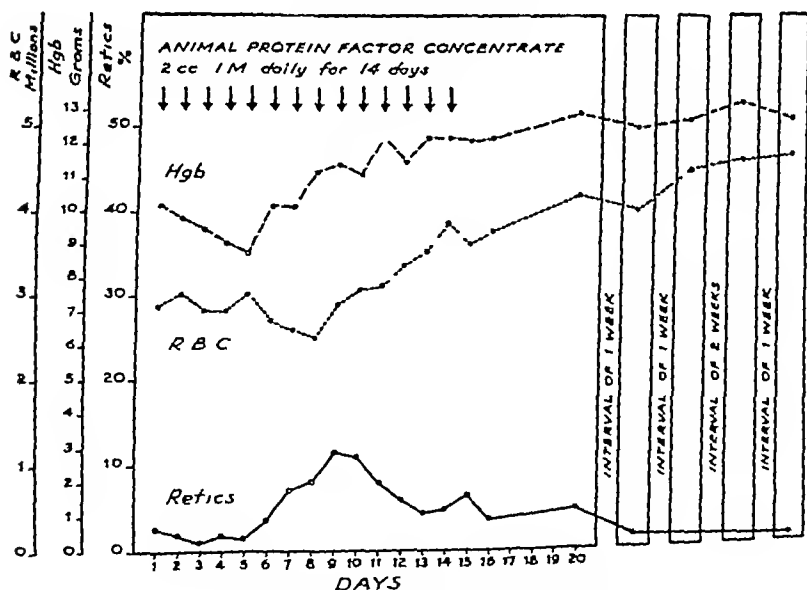


FIG 2—HEMOPOIETIC RESPONSE OF A PATIENT (E S) WITH NUTRITIONAL MACROCYTIC ANEMIA TO ANIMAL PROTEIN FACTOR CONCENTRATE

Case 2 E S a 78 year old white man had been under observation since 1943 at which time a diagnosis of nutritional macrocytic anemia was made. Following treatment with liver extract he had had

an excellent hematologic response and the mild glossitis that was present prior to therapy disappeared. He continued to eat a very inadequate diet as he had done for several years, worked every day, and did not receive maintenance therapy. His anemia relapsed each year for the following four years and each time was accompanied by a moderately severe glossitis. Liver extract administered at the time of each relapse was followed by a good hemopoietic response and relief of the glossitis. In January, 1948, the anemia relapsed again and he had a recurrence of mild glossitis. Following the administration of 10 mg. of folic acid by mouth daily for fifty-seven days, he showed an excellent hematologic response and the glossitis gradually disappeared. He returned to his former way of life with the result that in August he had a relapse of the anemia and a recurrence of the glossitis. The glossitis disappeared and a remission of the anemia was effected following a single injection of 25 micrograms of vitamin B<sub>12</sub>. The red blood cell count rose to 3.21 million and the hemoglobin determination was 10.2 grams (67 per cent). Further vitamin B<sub>12</sub> was not available and five weeks later the blood values began to decrease and the glossitis recurred. He lost

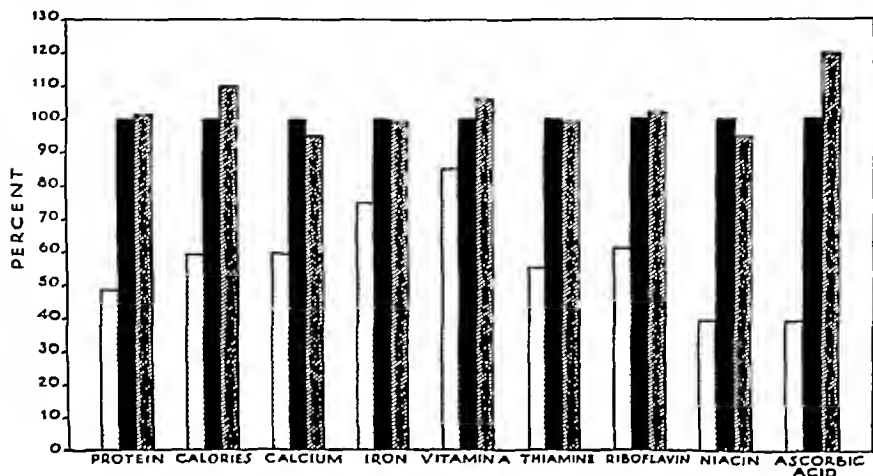


FIG. 3.—NUTRIENTS SUPPLIED BY DIET OF PATIENT WITH MACROCYTIC ANEMIA (E S) BEFORE AND AFTER THERAPY WITH ANIMAL PROTEIN FACTOR, CONTRASTED WITH RECOMMENDED ALLOWANCES OF NUTRIENTS (recommended by Council on Foods and Nutrition National Research Council)

Dotted columns: nutrient supplied by diet of patient before treatment. Solid black columns: recommended allowance of nutrient. Diagonally shaded columns: nutrient supplied by diet of patient after treatment.

his appetite and complained of weakness. At this time he was given 2 cc. of animal protein factor concentrate intramuscularly daily for fourteen days. In figure 2, which shows his hemopoietic response, it can be seen that the reticulocytes reached a peak of 11.6 per cent on the ninth day of therapy. By the last day of therapy, the red blood cell count had increased from 2.87 million to 3.88 million, the white blood cell count from 7,700 to 9,100, and the hemoglobin from 10.2 grams (67 per cent) to 12.2 grams (79 per cent). The hematologic response was accompanied by a great improvement in the patient's appetite and food intake (fig. 3) and the glossitis disappeared four days after therapy was initiated.

**Case 3: B. B.**—a 47-year-old Cuban woman with tropical sprue was treated with folic acid at the General Calixto García Hospital, Havana, Cuba, in June 1947. She had an excellent hematologic and clinical response at this time but, following her discharge from the hospital, she resumed eating a diet similar to that she had eaten for many years. It consisted chiefly of rice, cornmeal, bread, viandas (Cuban root vegetables), coffee, and sugar. She failed to return for follow-up studies but finally appeared at the hospital in December 1948 when she had moderately severe glossitis and diarrhea. Her appetite was very poor and she was so weak that she could do little of her housework. Her blood values were: red blood cells 2.01 million, white blood cells 3,750, hemoglobin 10.6 grams (69 per cent), and reticulocytes 0.8 per cent.

She was given an injection of 1 cc of animal protein factor concentrate. Three days later she volunteered that she felt stronger and that her appetite had improved. By this time the redness of the tongue had faded considerably and the tongue was less painful. Seven days after the injection the reticulocytes reached a peak of 8.0 per cent (fig. 4). Five days later the red blood cells increased to 2.16 million, the white blood cells to 5,050, the hemoglobin to 11.0 grams (71 per cent), the diarrhea was less severe. The following day she again complained of burning of the tongue, which showed increased redness along the border and at the tip. At this time, 1 cc of animal protein factor concentrate was injected and this amount was given every other day until a total of 4 cc were given. The glossitis began to subside three days after therapy was initiated. The reticulocytes reached a peak of 9.2 per cent seven days after the initial injection of animal protein factor concentrate and by this time the diarrhea and the glossitis had disappeared.

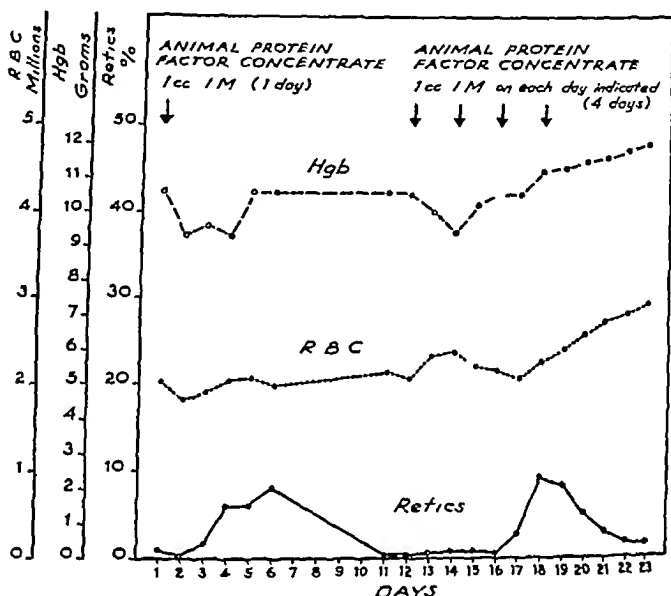


FIG. 4.—HEMOPOIETIC RESPONSE OF PATIENT (B. B.) WITH TROPICAL SPRUE TO ANIMAL PROTEIN FACTOR CONCENTRATE

Five days later the red blood cell count was 3.00 million, the white blood cell count 7,000, the hemoglobin 12.1 grams (78 per cent) and the reticulocytes 1.2 per cent.

**Case 4.** A R. a 68 year old white woman came to the Nutrition Clinic, Birmingham, Alabama in April 1948 complaining of severe soreness of the tongue which had persisted for over a year and varied in severity from time to time. It was so sore at times that she had difficulty in eating food of any kind particularly fruit and acid foods. She had severe general stomatitis and glossitis involving all the mucous surfaces of the oral cavity including the gums. The blood values were red blood cells 4.56 million, hemoglobin 15 grams (97 per cent). Repeated gastric analyses showed no free hydrochloric acid in the gastric juice after histamine stimulation. The patient came to the Clinic frequently for observation during the next six months and throughout this time the blood values and the glossitis remained about the same. She was then given 2 cc of animal protein factor concentrate intramuscularly daily for three days and she came to the Clinic daily for observation and blood examinations. Each injection was followed by local pain which lasted for about one hour. Seventy-two hours after the first injection there was some decrease in the soreness and burning of the mouth and tongue and they were considerably less red. The

injections of animal protein factor concentrate were discontinued for four days, during which time no further improvement to the glossitis and stomatitis occurred. Then the injections were resumed in the same amounts for four days. After the second, third, and fourth doses, the patient complained of pain at the site of the injection which persisted for twenty-four hours. Examination showed areas of about 10 cm in diameter which were red, tender, and slightly swollen. The pain and tenderness in these areas increased and the injections were discontinued at the end of four days. By this time the glossitis and stomatitis had disappeared. A subsequent intradermal test with a 1 to 20 solution of the concentrate gave a strongly positive reaction which developed rapidly within the first hour. At the end of twenty-four hours an area of swelling and redness with a central area of induration and tenderness of about 10 cm in diameter remained. After forty-eight hours there was a residual area of induration and swelling at the site of the skin test.

### DISCUSSION

Since the isolation of vitamin B<sub>12</sub> about a year ago, its function has been shown to be interwoven with many chemical substances. Yet the scientific story about it really begins with the findings of Minot and Murphy, which led to the inevitable conclusion that there was an active factor existing in liver and that this factor was a specific therapeutic agent against pernicious anemia. After the isolation of vitamin B<sub>12</sub>, it was found that this antianemic substance for persons had animal protein factor activity as tested on chicks. A number of micro-organisms are capable of synthesizing vitamin B<sub>12</sub> and probably related chemical substances. For some time to come there will be much study and speculation on the chemical identities of these various animal protein factor concentrates. At the present time the limited amount of clinical, biologic, and chemical evidence available in studying animal protein factor might suggest that this substance is identical with vitamin B<sub>12</sub>. Yet a more complete evaluation is needed, and it likely will prove that once again we are dealing with a complex of chemical compounds.

### SUMMARY AND CONCLUSIONS

The intramuscular injection of animal protein factor concentrate to 5 cases of pernicious anemia in relapse, 4 cases of nutritional macrocytic anemia in relapse, and 3 cases of tropical sprue in relapse was followed by a positive hematologic response in each case as is illustrated in figures 1, 2, and 4, respectively. The parenteral administration of this material to 3 patients with nutritional glossitis unassociated with anemia was followed by the disappearance of the redness and soreness of the tongue.

### REFERENCES

- <sup>1</sup> Woods R. The animal protein factor. *Borden's Review of Nutrition Research* 9: 1-12, 1948.
- <sup>2</sup> Hammond J. C. Dried cow manure and dried rumex contents as a partial substitute for alfalfa leaf meal. *Poultry Sc.* 23: 471, 1944.
- <sup>3</sup> Whitson D., Hammond J. C., Titus H. W., and Bird H. R. The use of soybean meal in the diet of growing chicks. II. Effect of different grains. *Poultry Sc.* 24: 408, 1945.
- <sup>4</sup> Bird H. R., Rubin M., Whitson D., and Haynes S. K. Effectiveness of high dietary supplements in increasing hatchability of eggs and viability of progeny of hen fed a diet containing a high level of soybean oil meal. *Poultry Sc.* 25: 285, 1946.
- <sup>5</sup> Mishler D. H., Carrick C. W., and Hauger, S. M. Condensed fish solubles as a supplement for corn and soybean oil meal chick rations. *Poultry Sc.* 28: 263, 1948.
- <sup>6</sup> Rubin M., Bird H. R., and Rothchild I. A growth promoting factor for chicks in the feces of heos. *Poultry Sc.* 25: 526, 1946.

- <sup>7</sup> ROSS, O B PHILLIPS P H AND BOHSTEDT G The effect of a simplified diet upon reproduction and lactation in swine and in the rat *J Animal Sc.* 1 86, 1942.
- <sup>8</sup> CUNHA T J A study of nutritional deficiencies of corn-soybean oil meal rations for swine and rats Ph D Thesis University of Wisconsin 1944.
- <sup>9</sup> SPITZER, R. R. AND PHILLIPS P H Reproduction and lactation studies with rats fed natural rations *J Nutrition* 32 631 1946
- <sup>10</sup> CARY, C A, HARTMAN, A M DRYDEN, L P AND LILEY G D An unidentified factor essential for rat growth *Fed Proc* 5 128, 1946
- <sup>11</sup> ZUCKER, L. M. ZUCKER T F BABCOCK B AND HOLLISTER P Zoopherin A nutritional factor for rats associated with animal protein sources *Arch Biochem* 26 115 1948
- <sup>12</sup> HEUSER G F, NORRIS L C LUCAS H L, AND COMBS, G F Further studies on the need by poultry for an unidentified factor associated with animal protein *Poultry Sc.* 25 403 1946
- <sup>13</sup> JOHNSON E L, CARRICK, C. W, AND ROBERTS R E. Evidence of new growth factors for chicks *Poultry Sc* 21 566 1942.
- <sup>14</sup> RICKES E. L., BRINK, N G, KONIUSZY F R WOOD T R. AND FOLKERS K Crystalline vitamin B<sub>12</sub> *Science* 107 396 1948
- <sup>15</sup> SMITH E L. Purification of anti pernicious anaemia factors from liver *Nature* 161 638, 1948
- <sup>16</sup> OTT W H RICKES E. L. AND WOOD, T R Activity of crystalline vitamin B<sub>12</sub> for chick growth *J Biol Chem* 174 1047 1948
- <sup>17</sup> STOKSTAD E L. R., PAGE A. JR PIERCE J FRANKLIN A L, JUKES, T H. HEINLE R. W ERSTEIN, M., AND WELCH A D Activity of microbial animal protein factor concentrates in pernicious anemia *J Lab & Clin Med* 33 860 1948
- <sup>18</sup> SPIES T D Experiences with Folic Acid Chicago, The Year Book Publishers, Inc, 1947

## PERNICIOUS ANEMIA AND RELATED ANEMIAS TREATED WITH VITAMIN B<sub>12</sub>

By EDGAR JONES, M D , WILLIAM J DARBY, M D , PH D , AND  
JOHN R TOTTER, PH D

IN APRIL, 1948, there appeared two reports of the isolation<sup>1</sup> or concentration<sup>2</sup> from liver of red crystalline substances which were hemopoietically active in pernicious anemia West<sup>3</sup> found that the material isolated in the Research Laboratories of Merck and Company was hemopoietically active in doses of the order of a few micrograms Shorb,<sup>4</sup> in collaboration with the Merck workers, found that this material served as an essential growth factor for *Lactobacillus lactis* Dorner This led to the microbiologic designation of LLD factor For general use, however, the term vitamin B<sub>12</sub> has been adopted<sup>1</sup> The structure of this new vitamin remains unknown It has been announced<sup>5</sup> <sup>6</sup> that it contains cobalt, phosphorus, and nitrogen and that it has a molecular weight of approximately 1600 It seems likely that the active red pigment from proteolyzed liver<sup>7</sup> is identical with vitamin B<sub>12</sub> West<sup>3</sup> found that 3 patients with pernicious anemia in relapse exhibited good reticulocyte responses followed by increases in red blood cells, hemoglobin, and volume of packed red cells after treatment with single initial doses of 150 µg , 6 µg , and 3 µg of the crystalline vitamin B<sub>12</sub>, respectively

Smith<sup>2</sup> has obtained two red pigments, both highly active in pernicious anemia, from ox liver Proteolyzed liver extract was the source of the more potent materials<sup>2</sup> <sup>7</sup> Separation by partition chromatography gave preparations which were effective in pernicious anemia in 0.3 mg doses It was known that these materials were not pure Their clinical efficacy appeared to be directly proportional to the color intensity Ten batches of material had been found to have clinical activity in 26 cases of pernicious anemia It was also stated<sup>2</sup> that these pigments were effective in 3 cases of subacute combined degeneration of the spinal cord No clinical data were provided

The materials obtained by the British investigator were described as very soluble in water, but insoluble in ether or chloroform Smith concluded that they had obtained two differing forms of the liver factor effective in pernicious anemia and suggested that only this one factor is required for both the hematologic and neurologic aspects

A recent report<sup>8</sup> by the Merck group reveals that a number of sources of vitamin B<sub>12</sub> have been discovered Among these are milk powder, beef extract and culture broths of several micro-organisms Of special interest is the finding that *Streptomyces griseus*, from which streptomycin is obtained, is a source of vitamin B<sub>12</sub>

Recently Berk, Denny-Brown, Finland and Castle<sup>9</sup> have reported great neuro-

From Departments of Medicine and Biochemistry Vanderbilt University School of Medicine Nashville Tenn and Department of Biochemistry University of Arkansas School of Medicine Little Rock Ark

This work was supported in part by grants from National Vitamin Foundation Incorporated Nutrition Foundation Incorporated International Health Division of the Rockefeller Foundation

logic and hematologic improvement following the giving of vitamin B<sub>12</sub> to a patient who had experienced a hematologic relapse and developed severe neurologic damage despite therapy with pteroylglutamic (folic) acid alone. Their patient who had shown sensitivity to various liver extracts had no such reaction to vitamin B<sub>12</sub>.

Spies and co-workers,<sup>10</sup> in observations extending over a fourteen day interval, have confirmed the initial finding by West that vitamin B<sub>12</sub> has hemopoietic activity in pernicious anemia. They have also indicated that it is hemopoietically active in both sprue<sup>11, 12</sup> and nutritional macrocytic anemia.

The present report presents observations on 8 patients with pernicious anemia, one with sprue, one with nutritional macrocytic anemia, and one with anemia secondary to the absorptive defect of intestinal lipodystrophy, who have been treated with crystalline B<sub>12</sub> (Merck) for periods up to six months. The vitamin B<sub>12</sub> which

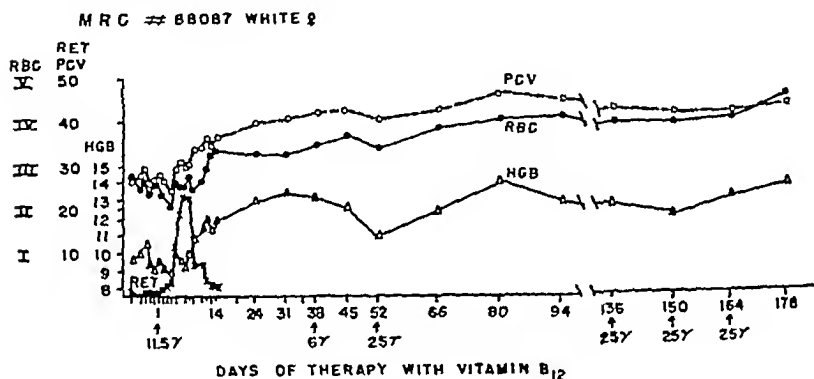


CHART 1

we used was generously supplied through the courtesy of Doctor Augustus Gibson of Merck and Company.

The following case summaries indicate the background of these patients.

#### CASE REPORTS

##### *Pernicious Anemia*

Each of these patients presented typical hematologic and marrow findings of pernicious anemia, all had histamine refractory achlorhydria, and absence of roentgenologic evidence of gastrointestinal defects except where specifically mentioned. Neurologic changes were absent except where specifically mentioned. None of the patients had diarrhea or other findings suggestive of sprue.

1. M. R. C. white female age 67 was admitted to Vanderbilt University Hospital in October 1937 with typical findings of pernicious anemia. She responded well to treatment with liver extract administered parenterally. Treatment with liver extract was continued regularly until October 1945 when it was deliberately withdrawn in order to observe the time necessary for relapse.<sup>13</sup> By May 23, 1948, relapse sufficient to permit evaluation of treatment with vitamin B<sub>12</sub> had occurred. The details of the hematologic response are shown in Chart 1. Pronounced symptomatic improvement has also been noted.

2. F. L. colored male age 45, was admitted to the Vanderbilt University Hospital in August, 1937, in an almost comatose state alternating with periods of mild delirium. His red blood cell count was 460,000 with 1.5 Gm of hemoglobin. He was treated with blood transfusions and parenteral liver extract. Two months after his admission to the hospital his red blood cell count was 3.3 million and hemoglobin 7.9 Gm. He was not seen again until two years later when he returned in relapse. He had taken liver extract injections quite irregularly and had eaten liver only occasionally. His red blood cell count was 1.75 million with 6.7 Gm of hemoglobin at this time. He was started again on therapy with liver extract which was followed by an excellent response. Liver extract injections were continued at intervals of three weeks in amounts of 30 units per injection until December 1, 1945, when it was deliberately withdrawn in order to observe the time of relapse.<sup>12</sup> By June 3, 1948, his red blood cell count had fallen to 2.3 million with 10.1 grams of hemoglobin and he was readmitted to the hospital for treatment. He was given vitamin B<sub>12</sub> with hematologic results shown in Chart 2. Following therapy with B<sub>12</sub> there has been a great increase in his sense of well being.

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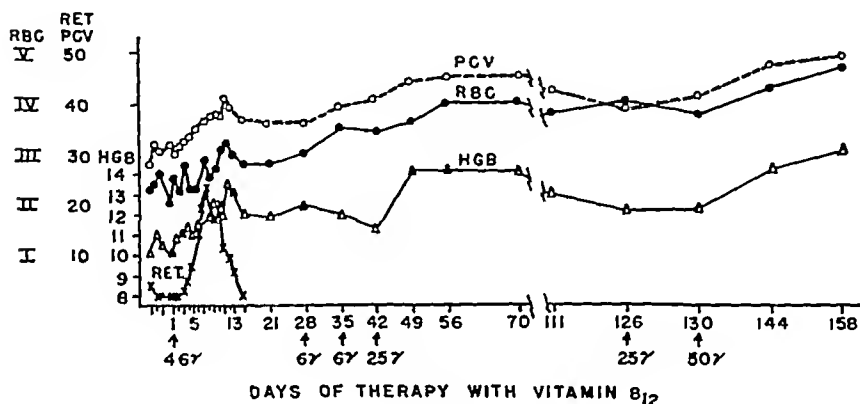


CHART 2.

3. A. J., white male age 61 was admitted to Vanderbilt University Hospital in March 1936 with the full blown picture of pernicious anemia. Regular administration of liver extract parenterally resulted in an excellent response and had maintained satisfactory blood levels without development of neurologic symptoms. In November of 1945 liver extract was discontinued in order to permit relapse.<sup>12</sup> His blood values fell slowly until on June 12, 1948 at which time his red blood cell count was 2 million and he was admitted to the hospital for treatment. Chart 3 shows the hematologic response to vitamin B<sub>12</sub>. Coincident with the return of hematologic values to normal there was a great improvement in the sense of well-being. He has resumed his work as a carpenter.

4. O. C. white male age 50 was admitted to Thayer Hospital in December 1947 with typical blood and marrow findings of pernicious anemia. He had had a sore tongue and intermittent diarrhea during this time. He was treated with pteroylglutamic acid the hematologic values before and after treatment being shown in Chart 4. At the time of discharge from the hospital the folic acid was discontinued and injections of liver extract were advised. He discontinued all therapy and was readmitted in a hematologic relapse in July 1948. He complained of numbness and tingling of his hands and feet and there was some disturbance in his position sense and absence of vibratory sense over his ankles. The hematologic course is indicated in Chart 5. Within four weeks from the start of therapy with vitamin B<sub>12</sub> there had been a return of vibratory sense over the malleoli and complete disappearance of the paresthesias.

5. E. B. M. white male age 52 was admitted to Thayer Hospital August 13 1948. A diagnosis of pernicious anemia had been made six months earlier and he had been treated inadequately with liver



extract The hematologic findings at the time of admission and following treatment with vitamin B<sub>12</sub> are recorded in Chart 6 He gained 8 pounds in weight during his hospital stay and was greatly improved generally

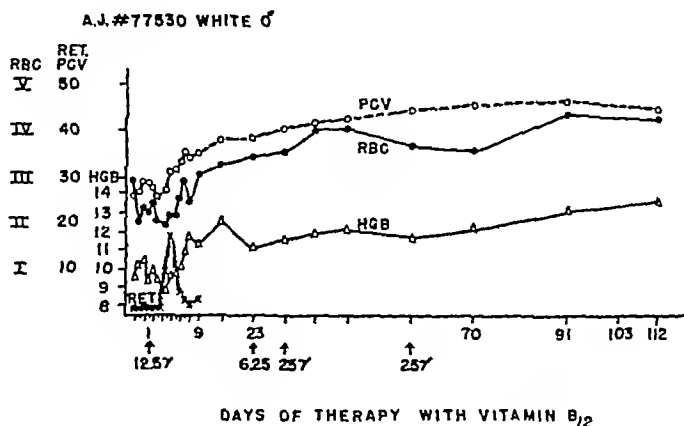


CHART 3

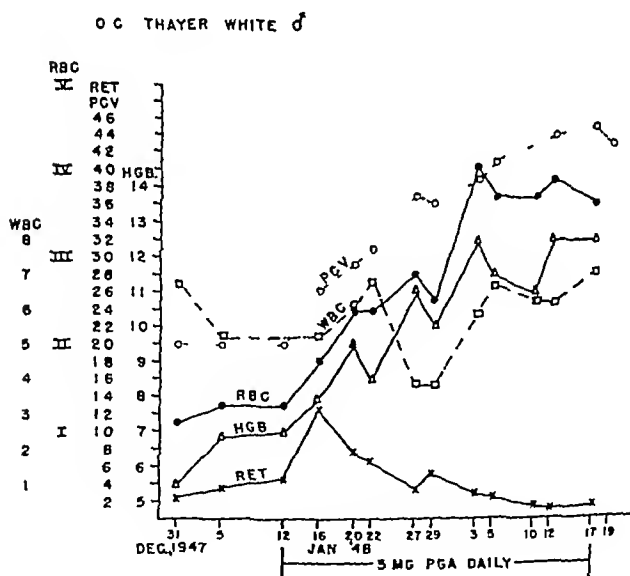


CHART 4

6 A M., white male, age 43 entered Thayer Hospital on July 14 1948 where he was found to have typical findings of pernicious anemia For six months he had noted a burning tingling sensation associated with numbness in both hands and both feet. There was no equivocal Babinski and vibratory sense was absent over the legs below the knees A neurologic consultant concurred in the diagnosis of mild

## OC THAYER WHITE ♂

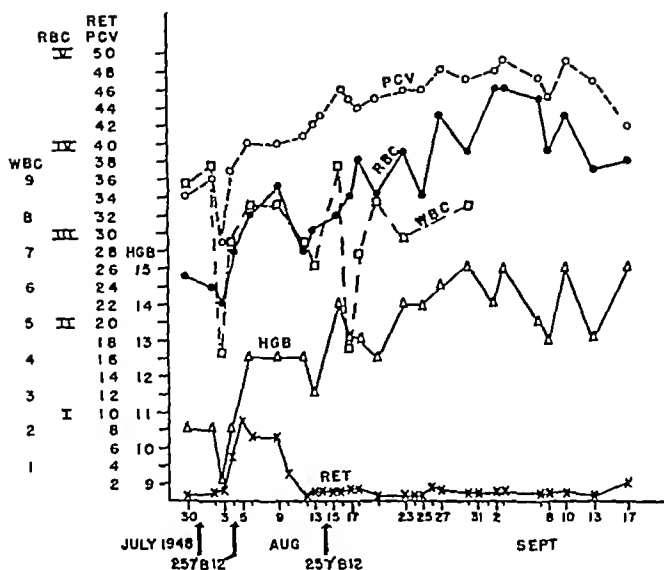


CHART 5

## ME THAYER WHITE ♂

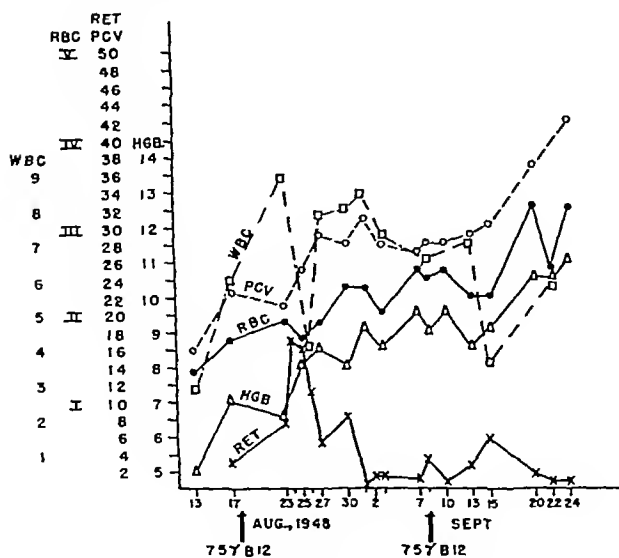


CHART 6

combined system disease. The hematologic values before and after treatment with vitamin B<sub>12</sub> are shown in Chart 7. By three weeks after institution of therapy vibratory sensation over the lower extremities had partially returned and he no longer experienced the paresthesias. General symptomatic improvement was satisfactory.

7 W. H. M., white male, age 73, was admitted to Vanderbilt University Hospital August 3, 1948, with the findings of pernicious anemia. Glossitis and paresthesias had been particularly bothersome. Hematologic values before and after treatment with vitamin B<sub>12</sub> are shown in Chart 8. Treatment with B<sub>12</sub> has been followed by much symptomatic improvement including relief of the glossitis and paresthesias.

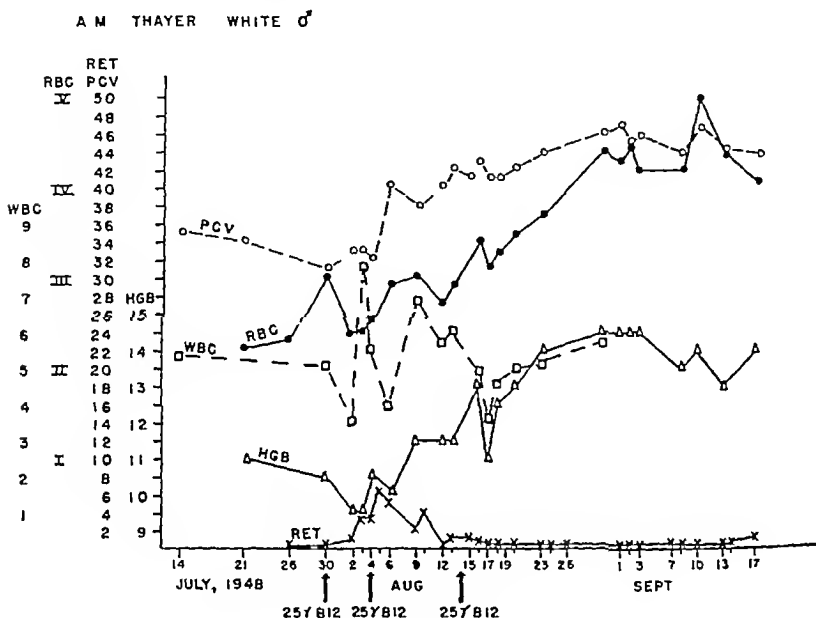


CHART 7

8 L. K., a white housewife, age 45, entered the Vanderbilt University Hospital in 1936 with the complete hematologic picture of pernicious anemia. Administration of parenteral liver extract was followed by rapid relief of her symptoms. She was not seen again until October 1948, when she returned in severe hematologic relapse. During the twelve year interim she had received little, if any, therapy. In addition to the hematologic findings, there was diffuse atrophy of the lingual papillae. She was given 10 µg of vitamin B<sub>12</sub> parenterally daily for twenty-two days. The hematologic response is shown in Chart 9. Coincident with this response she became much more alert mentally, the glossitis cleared, and she gained approximately 4 pounds in weight during the first month of treatment.

### Nutritional Macrocytic Anemia

E. P., a white female, age 44, was admitted to Vanderbilt University Hospital in 1944 with the complaint of nausea, vomiting, diarrhea, and weakness of two years' duration. There had been a weight loss of 64 pounds. Physical examination revealed a pale, emaciated woman with no glossitis or evidence of combined system disease. There was a normochromic anemia with 2.4 million red blood cells and 7.5 Gm of hemoglobin, and considerable variation in size and shape of the red cells. Free gastric acid was found after histamine injection. The BMR was -22 per cent of normal. Cysts of *E. histolytica* were

found in stools. Sternal bone marrow was generally hyperplastic in both the red and white cell series. No megaloblasts were noted. She was treated with liver extract transfusions of whole blood desiccated thyroid and carbasone. Upon discharge from the hospital four weeks after admission her red cell count

## WM #10730 WHITE ♂

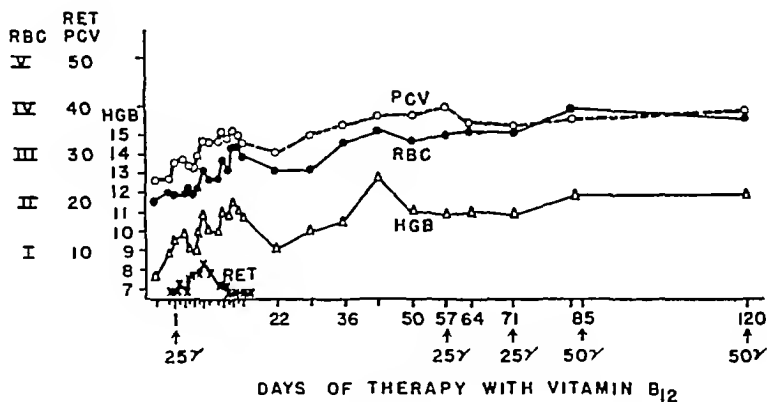


CHART 8

## K #79682 WHITE ♀

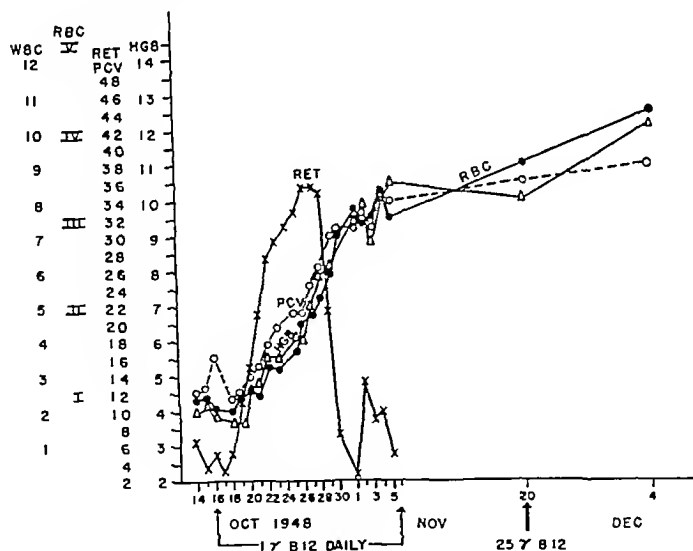


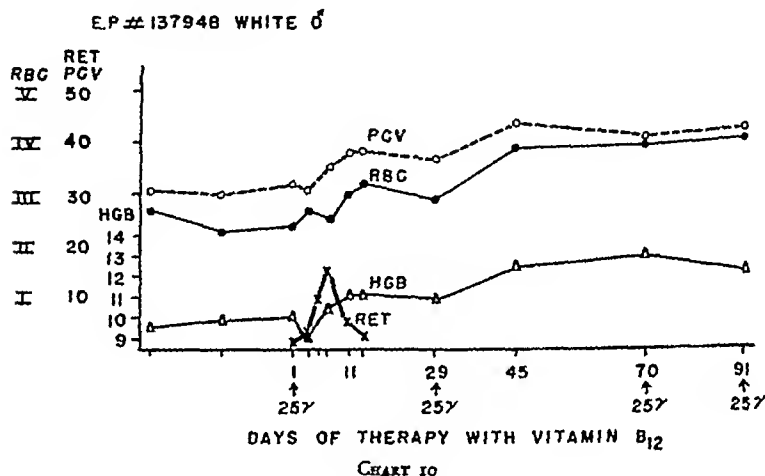
CHART 9

was 3.5 million with 10.7 Gm of hemoglobin. There was considerable symptomatic improvement. Liver extract was discontinued but the thyroid medication was continued.

Three months later she returned to the hospital in a profound relapse. Her red cell count was 1.0 million, hemoglobin 4.0 Gm, and white cell count 1,350. The mean corpuscular volume was again within upper normal limits. Sternal bone marrow showed numerous megaloblasts.

She was given two transfusions followed by 260 units of liver extract over a period of twenty-seven days with a 31 per cent reticulocyte response. By four weeks her red cell count was 3.79 million with 11.0 Gm of hemoglobin. There was great symptomatic improvement. Liver extract was continued for six months and then deliberately discontinued. One year later she had no anemia. A cholecystectomy was performed. Thirty-two months after the last injection of liver extract there were decreased hematologic values, and bouts of diarrhea and glossitis were occurring. By September 1948, (thirty-five months after last therapy) her red cell count was 2.4 million, hemoglobin 9.5 Gm, packed red cell volume 31 per cent. She complained of increasing nocturnal diarrhea. Serum fat-soluble vitamins were: carotene, 33  $\mu$ g per cent, vitamin A, 117 international units, vitamin E, 0.56 mg per cent. The hematologic response to parenteral vitamin B<sub>12</sub> is shown in Chart 10.

Coincident with hematologic improvement there was rapid cessation of the diarrhea and general improvement.



### Sprue

P. B. This 67 year old white man entered Vanderbilt University Hospital on November 8, 1945, at which time the previously established diagnosis of sprue was confirmed and he was successfully treated with synthetic pteroylglutamate. The details of the course during that period were published as Case 3 of a previous report.<sup>14</sup>

Continued oral treatment with pteroylglutamic acid resulted in red cell values of around 3.5 million until January 1948 when a gradually increasing anemia developed. By May 1948 values had fallen to 1.6 million red cells, a packed cell volume of 23 per cent and hemoglobin of 7.6 Gm. Fat and sugar absorption had remained impaired throughout the period of observation. The hematologic decline was not accompanied by return of glossitis or diarrhea. He was then given 15 mg. of pteroylglutamic acid daily by injection with a slight reticulocyte response but with little red cell increase. He was then treated with vitamin B<sub>12</sub>. Hematologic response is shown in Chart 11. This was followed by a second reticulocyte peak slightly higher than had been observed after pteroylglutamic acid. A slight gradual erythropoiesis followed. The red cell count has now stabilized at an average level of less than 3.0 million and macrocytosis has persisted.

### Conditioned Anemia with Megaloblastic Arrest

Mrs. R. D., a 36 year old white woman entered Vanderbilt University Hospital on September 8, 1948 with the complaints of diarrhea, weight loss, and anorexia beginning ten to twelve years before. She had lost about thirty pounds during this period. Her symptoms had gradually increased until she was now

having eight to ten semiliquid stools daily. Glossitis had been noted for some months prior to admission. Tetany had occurred occasionally during the past three years. Physical examination showed emaciation,

P B #98144 WHITE ♂

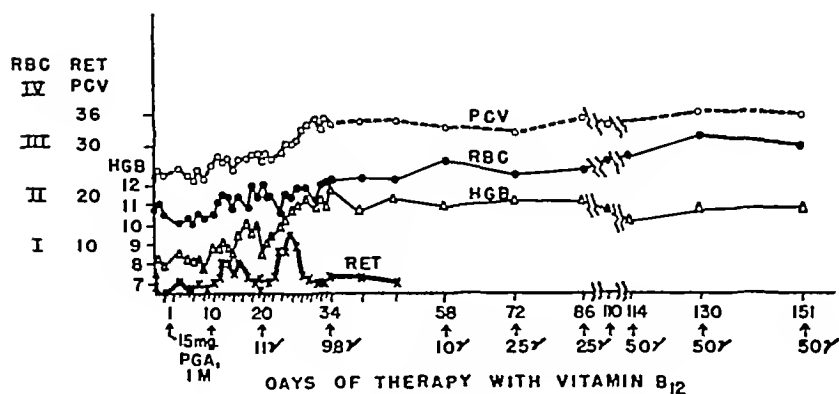


CHART 11

R D #172788 WHITE ♀

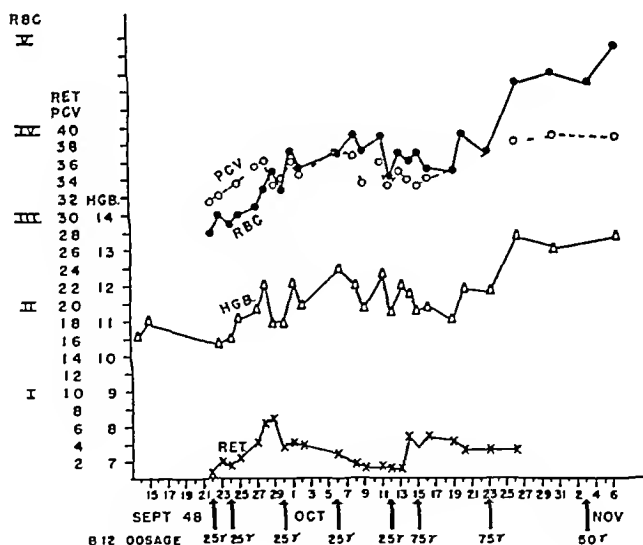


CHART 12

papillary atrophy of the tongue and a positive Chvostek sign. There was no evidence of neurologic disease.

Laboratory studies revealed a red count of 3.0 million, hemoglobin of 10.5 Gm, PCV 32.0 per cent, reticulocyte count of 2.0 per cent, a histamine refractory achlorhydria, serum proteins 5.10 Gm. per 100 cc., albumin 3.75 Gm, serum calcium 7.3 mg per cent, phosphorus 3.2 mg per cent, serum vitamin C 0.24

mg per cent, serum carotene, vitamin A and tocopherol levels were respectively 36.0  $\mu$ g per cent, 122 I U per cent, and 0.54 mg per cent. An oral glucose tolerance test showed a maximum rise of 16.0 mg per cent above fasting, and a vitamin A tolerance curve following oral ingestion of 200 000 I U was as follows: fasting 107 I U, three hours 148 I U, five hours 220 I U, ten and one-half hours 162 I U. Quantitative determination of stool fat content revealed 22.5 per cent of dry weight. Marrow showed 2.0 per cent megaloblasts. Roentgenologic examination revealed coarsening of the small bowel mucosal pattern with clumping of barium and hypermotility.

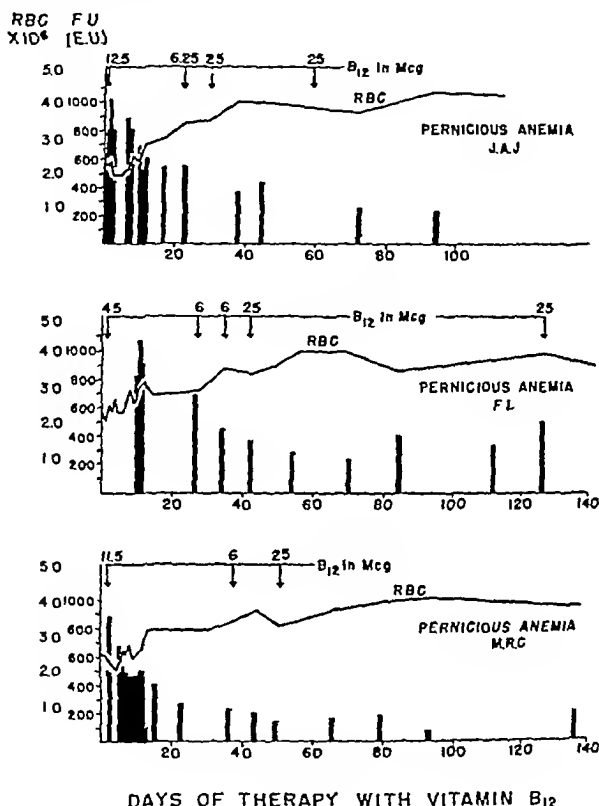


FIG 1

The history and findings were thought to be compatible with either primary or secondary defective gastrointestinal absorption associated with a deficiency of hemopoietic factors. Accordingly the patient was treated with vitamin B<sub>12</sub> as indicated in Chart 12. The depicted hematologic improvement resulted accompanied by a decrease in the number of stools from six to eight per day to about three per day, a weight gain of 4 pounds within seven days associated with minimal ankle pitting and complete relief of the glossitis.

Four weeks after the initial therapy with vitamin B<sub>12</sub> she developed epigastric pain, distention and vomiting. X-ray study showed a marked narrowing of the lower ileum suggesting regional enteritis with a deficiency pattern in the remaining portion of the small intestine. Surgical exploration was carried out the small bowel being found to be remarkably swollen and thickened with a gelatinous appearance due to extensive edema. There was no inflammatory reaction in the adjacent edematous mesentery. Large

moderately indurated nodes were found at the root of the mesentery and scattered white nodules were seen throughout the mesentery. Microscopic examination of these nodes revealed a picture compatible with intestinal lipodystrophy or Whipple's disease.

### METABOLIC STUDIES

Fecal urobilinogen estimations<sup>15</sup> were made on random stool specimens from most of the patients with pernicious anemia. In all cases of pernicious anemia

TABLE 1—*Urinary Excretion of Pteroylglutamates and of Porphyrin by Patients Treated with Vitamin B<sub>12</sub>*

Day of therapy	Pernicious anemia												Sprue	
	Case 1		Case 2		Case 3		Case 7		Case 6		Case 4		P.B.	
	PGA	Por†	PGA	Por	PGA	Por	PGA	Por	PGA	Por	PGA	Por	PGA	Por
-9													15,250	84.8
-8													31.7	86.5
-7													12.0	38.9
-6													21.7	51.6
-5													62.5	21.2
-4	1.2	21.2											0.0	35.2
-3	2.8	10.6											0.0	8.7
-2	0.0	8.4	0.0	15.2									0.0	10.6
-1	1.0	5.4	0.0	17.4	0.0	34.0							6.4	14.3
1	0.0	10.6	3.3	34.8	0.0	28.7	5.8	30.0					3.4	10.9
2	0.0	6.4	3.4	28.9	0.0	35.0	0.0	8.2	2.0	19.3	1.3	12.0	3.1	13.2
3	0.0	10.0	0.0	37.0	0.6	43.1			2.7	10.3	1.4	16.0	6.2	11.5
4	0.0	16.7	0.0	30.2	0.0	72.1	0.0	5.2	1.6	4.3	2.5	26.9	4.0	16.1
5	0.0	10.5	3.3	7.0			0.0	12.8	12.3	106.0	0.2	4.3	5.5	60.9
6	0.0	2.7	1.3	25.1	1.6	43.2	0.0	9.6	1.0	10.4	0.0	30.4	7.0	54.6
7	0.0	3.7	3.5	64.1	0.8	46.1	0.0	4.8	1.5	12.8	0.0	18.6	8.4	28.3
8	0.0	5.9	0.0	9.8	0.0	50.5			0.0	12.7	0.0	21.6	6.7	29.9
9	0.0	4.3	1.9	58.6	0.8	36.9			0.0	10.9	2.8	14.6	5.0	13.2
10	0.0	4.8	0.0	49.6	0.0	43.2	0.0	3.2	7.9	3.0	0.6	4.3	4.1	38.7
11	1.0	5.8	0.0	43.0	0.0	34.8	0.0	4.0	0.0	4.6	1.7	13.8	4.3	85.4
12	0.0	1.9	0.0	31.4	1.0	23.2	0.0	4.4	0.0	11.2	1.3	14.8	5.2	96.9
13	0.0	5.4	1.5	24.2			0.0	2.4	0.0	12.6	2.0	7.3	3.9	81.2
14	0.0	37.3	0.0	22.2					1.2	6.1	1.1	13.4	3.5	66.0
15	0.0	1.7	0.0	22.2					0.0	8.4	1.8	20.8	3.2	44.7
16			1.2	34.0					2.0	49.4	1.1	10.0	5.8	45.3
17									2.3	11.3	1.1	15.4		

\* In each case,  $\mu\text{g}/\text{Gm}$  creatinine. Concentrations of PGA of less than 0.5  $\mu\text{g}/\text{l}$  are reported as 0.0.

† In each case, units/Gm creatinine. One unit of porphyrin is defined as the extinction at 402  $\mu$  (1 cm.)  $\times$  volume.

increased values were found during relapse and these decreased upon treatment. Three illustrative cases are presented in figure 1.

Total 24-hour collections of urine were made on selected patients prior to and during the first several days of treatment. The following were determined: creatinine, total pteroylglutamic acid by a microbiologic assay, and unidentified urinary porphyrins.<sup>16</sup> These data are presented in table 1.



## DISCUSSION

Vitamin B<sub>12</sub> is the third chemically distinct substance which has been demonstrated to possess hemopoietic activity in those anemias which are characterized by megaloblastic arrest. The first group of these substances was the pteroylglutamates—the physiologic action of which has been reviewed elsewhere.<sup>17-18</sup> The second substance, thymine, was found by Spies and co-workers<sup>19</sup> to be hemopoietically active in large amounts in pernicious anemia and sprue. The preliminary nature of the reports which have appeared to date has precluded any comparative studies on the completeness of the hemopoietic response to vitamin B<sub>12</sub> or of other effects of this newly isolated substance. Furthermore, the dosages employed, ranging from 3 to 150  $\mu$ g of crystalline material, have permitted only an approximation of the minimal effective dose, for either initial response or maintenance.

Our observations on these 11 patients who have been treated with vitamin B<sub>12</sub> permit certain generalizations. Single injections of as little as 4-6  $\mu$ g to a patient with pernicious anemia may be followed by a reticulocyte response which approximates the standard response expected from liver<sup>20</sup> (Case 2). None of our patients who received less than 50 or 75  $\mu$ g of vitamin B<sub>12</sub> at a single injection during the initial phase of therapy has attained erythrocyte levels which could be termed satisfactory until additional therapy has been given. The nature of the erythrocyte responses in patients 1 and 2 indicate that the rate of utilization of the vitamin in patients with pernicious anemia approximates 10  $\mu$ g per day. This statement is based on the observation that single small injections were followed by attainment of submaximal erythrocyte levels and then decreases in red cell counts unless additional B<sub>12</sub> was administered. With increased amounts of therapy these patients then showed additional erythrocyte regeneration. In the first report<sup>1</sup> on vitamin B<sub>12</sub>, it was hypothesized that 10  $\mu$ g of the vitamin would have the approximate equivalence of 10 U.S.P. unit of injectable liver. Additional studies by West (personal communication) and Bethell and co-workers<sup>21</sup> have tended to bear out this approximation. The course of patient 8 in this series demonstrates that an excellent reticulocyte and erythrocyte response resulted from the injection of 10  $\mu$ g of B<sub>12</sub> daily. When these injections were discontinued after twenty-two days, hemo-regeneration did not continue until additional therapy was provided. Obviously, 10  $\mu$ g daily is not a quantity which will allow significant storage of the vitamin.

Table 2 presents a tabulation of the maximum reticulocyte responses in all of the patients with pernicious anemia who have been reported by others and those included in the present report. It is apparent that the maximum response is grouped about the average as expected from liver extract in a manner seemingly independent of size of dose. Reference to the charts of individual patients in this series demonstrates that attainment of the maximum reticulocyte response is not assurance that the quantity of therapeutic agent administered will support maximum hemo-regeneration. This again emphasizes the unreliability of the reticulocyte count<sup>22</sup> as a quantitative measure of activity of a substance or of the adequacy of therapy in a given patient.

Four of these patients had been treated with liver extract in a previous relapse. Upon withdrawal of liver extract they slowly relapsed.<sup>12</sup> A second remission was

then induced by vitamin B<sub>12</sub>. Table 3 shows comparative hematologic data for the two types of therapy. These observations indicate that less than maximum erythropoiesis is maintained by the parenteral administration of quantities of B<sub>12</sub> which average less than 0.75 µg daily.

Figure 2 relates the rate of erythropoiesis to dose of vitamin B<sub>12</sub> for 17 patients with pernicious anemia. This tabulation is based on 7 patients in the series here reported and 10 of the group reported by Hall and Campbell.<sup>18</sup> Only those patients are included in this tabulation whose reported count following treatment was 4.0 million or greater, and where possible, the calculation of dosage is based on the shortest interval between institution of therapy and the attainment of a sustained

TABLE 2.—*Comparison of Observed Maximum Reticulocyte Response of Patients with Pernicious Anemia following Therapy with Vitamin B<sub>12</sub> with the Maximal Response<sup>20</sup> following Treatment with Liver Extract*

Source of data	Initial RBC	Observed maximum reticulocytosis	Expected maximum reticulocytosis	Total dose of B <sub>12</sub> prior to peak
	millions	per cent	per cent	µg
Present report				
Case No. 1	2.50	22.5	12.0	11.5
Case No. 2	2.40	23.4	13.3	4.6
Case No. 3	2.50	17.0	12.0	12.5
Case No. 7	2.20	7.4	16.0	25.0
Case No. 8	0.90	35.7	42.9	11.0
Case No. 5	1.30	17.0	32.4	7.5
Case No. 6	2.20	8.0	16.0	50.0
Case No. 4	2.50	9.4	12.0	50.0
West <sup>3</sup>	1.50	27.0	28.0	150.0
	1.50	26.0	28.0	6.0
	1.40	10.2	30.1	3.0
Castle et al. <sup>9</sup>	1.90	16.0	20.6	30.0
Spies et al. <sup>10</sup>	2.37	12.8	13.0	6.0
	2.50	14.6	12.0	15.0

level above 4.0 million. We recognize that this calculation of the average rate of increase in red cell count does not permit adjustment for the known differences in rate of erythrocyte increases associated with different initial red cell levels. Furthermore, the calculation of average daily dose of B<sub>12</sub> as made here does not recognize possible differences in rate of excretion, utilization, or degradation of the vitamin which may occur when different sized dosages are administered. Nevertheless, the data indicate that the maximum rate of erythropoiesis will require in most cases more than 1.0 microgram of vitamin B<sub>12</sub> per day. The one patient from our series which exhibits a maximum rate of erythropoiesis on 1.0 µg of B<sub>12</sub> received daily injections and may have, thereby, utilized the material more efficiently. Hemopoiesis seems to be more consistently rapid in those patients receiving 2.5 micrograms or more of B<sub>12</sub> per day. These several considerations lead

us to make the tentative suggestion that a reasonable dosage schedule for vitamin B<sub>12</sub> in the treatment of pernicious anemia should provide approximately 30 micrograms of the vitamin daily during the first six weeks or so, and that a maintenance dose of 10 microgram per day thereafter does not appear unreasonable

TABLE 3—Comparison of the Response to Adequate Therapy with Liver Extract with the Response to Vitamin B<sub>12</sub> in a Subsequent Relapse

Diagnosis	Patient	Status	Therapy	RBC millions	Hemoglobin Gm./100 cc
Pernicious anemia	Case 3	Relapse		1 85	6 4
		Remission	Liver extract 66 days	4 90	12 7
		Relapse		2 50	9 9
		Remission	43 5 gamma B <sub>12</sub> 72 days 114 days	3 53 4 24	12 0 13 5
	Case 1	Relapse		3 1	12 0
		Remission	Liver extract 66 days Maximum	3 4 4 75	12 6 13 7
		Relapse		2 50	9 3
		Remission	42 5 gamma B <sub>12</sub> 63 days 117 5 gamma B <sub>12</sub> 176 days	3 68 4 40	12 2 13 5
	Case 2	Relapse		1 74	6 9
		Remission	Liver extract 53 days	4 96	14 2
		Relapse		2 40	11 2
		Remission	39 6 gamma B <sub>12</sub> 56 days 117 gamma B <sub>12</sub> 164 days	4 04 4 70	14 0 15 0
Nutritional macrocytic anemia	E. P	Relapse		1 53	5 0
		Remission	410 units liver extract 61 days Average maximum on liver	3 91 5 13	11 8 13 7
		Relapse		2 37	9 9
		Remission	50 gamma B <sub>12</sub> , 44 days	3 89	12 5

In addition to these hematologic observations, we have noted the expected disappearance of megaloblasts from the marrow of patients treated with vitamin B<sub>12</sub> and the appearance of numerous normoblasts in the marrow during the early phase of remission. The hemopoietic response to vitamin B<sub>12</sub> has been accompanied by a decrease in the fecal urobilinogen (fig. 1). It is known that liver extract promotes a similar reduction in fecal urobilinogen in the patient with pernicious anemia. We have observed like decreases in fecal urobilinogen in patients treated with folic acid. The exact interpretation of this observation, however, is not clear. In the

past such findings have been interpreted as indicating that the breakdown of red cells has decreased and, indeed, such an interpretation is consistent with new evidence<sup>44</sup> that pernicious anemia is a true hemolytic syndrome. It may be, therefore, that vitamin B<sub>12</sub> and PGA decrease the fecal urobilinogen of patients with pernicious anemia by promoting the formation of a more nearly normal erythrocyte. On the other hand, these findings might also reflect a decrease in urobilinogen formation due to a postulated effect of vitamin B<sub>12</sub> on some step in pigment metabolism quite apart from the breakdown of hemoglobin. London, Shemin, and Rittenberg<sup>45</sup> have demonstrated that a significant portion of the normal stercobilin production must come from sources other than hemoglobin. It may be, therefore,

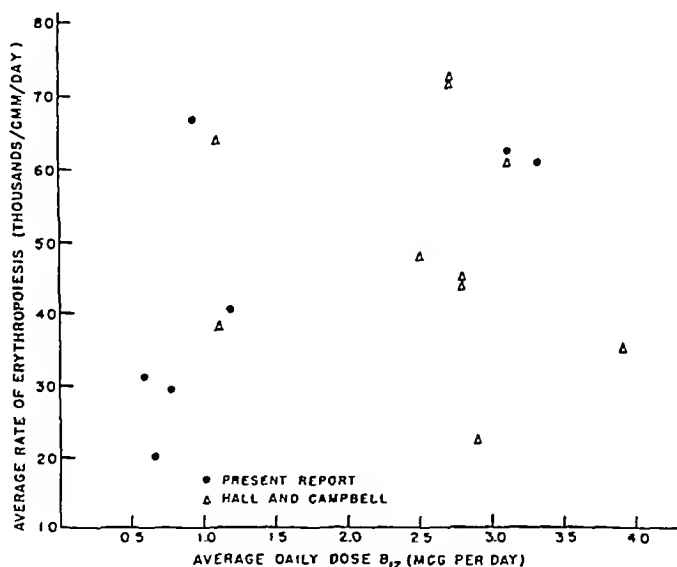


FIG. 2.

that the site of action of vitamin B<sub>12</sub>, PGA, etc., in pigment metabolism is on this step rather than in the production of a normal cell. Again, it may be that the different hemopoietic agents do not act at the same point. Studies of this possibility may aid in elucidating the paradox of two chemically distinct factors exhibiting like metabolic effects in the patient with pernicious anemia. Investigations of these possibilities are under way.

Vitamin B<sub>12</sub> administration had no effect on the urinary excretion of PGA in the six cases of pernicious anemia and the one case of sprue investigated (Table 1). This does not rule out a possible metabolic inter-effect of B<sub>12</sub> on pteroylglutamates, but it does suggest that administration of effective doses of B<sub>12</sub> does not result in a great release of PGA. It is to be noted that the patient with sprue had been saturated with PGA prior to the giving of B<sub>12</sub>. Even under this circumstance there was no increase in urinary loss of PGA attributable to the B<sub>12</sub>.

The urinary porphyrin values in arbitrary units are also included in table 1. It is obvious that no recurring pattern of porphyrin excretion in 24-hour specimens followed B<sub>12</sub> therapy. Since separate 2-hour collections were not made, it is impossible to state whether an increased excretion occurred at 2 to 4 hours after therapy such as has been noted following PGA.<sup>15</sup>

Our two patients with mild neurologic involvement improved while receiving vitamin B<sub>12</sub>. This observation confirms the reported experience of both Ungley (as quoted by Smith<sup>2</sup>) and Castle and co-workers<sup>9</sup> and indicates that vitamin B<sub>12</sub> is more nearly complete replacement for patients with pernicious anemia than is either thymine or folic acid.

The response of the single patient we have observed with nutritional macrocytic anemia was equally good to vitamin B<sub>12</sub> as to liver extract. In the patient with sprue, on the other hand, the evidence is not so clear-cut. This patient had initially exhibited a rapid response to synthetic folic acid, and although he had attained submaximal erythrocyte levels, these levels were equally as high as he had previously reached during a period of intensive treatment with liver extract. A hematologic relapse occurred while he was receiving presumably adequate therapy with PGA. The relative resistance of this patient to PGA was further attested by the finding that he showed very little response to administration of 15 mg of pteroylglutamate per day parenterally. Upon the subsequent administration of vitamin B<sub>12</sub>, the patient exhibited a definite reticulocytosis and a gradual increase in red cells, hemoglobin and packed cell volume. Over a period of twenty seven weeks, very large quantities of B<sub>12</sub> have been administered and, as yet, the patient has not attained as high erythrocyte count as he had previously reached on liver extract or on folic acid. A thorough study has revealed no complicating disease which would account for this incomplete response. These observations are compatible with the interpretation that this patient has become deficient in some additional hemopoietic factor during the two and one-half to three years of therapy with folic acid alone.

The patient with anemia associated with intestinal lipodystrophy is believed to represent a deficiency of the hemopoietic factor conditioned by the gastrointestinal defect. The absorptive defect was unaltered by the vitamin B<sub>12</sub> as was to be expected when the true nature of the defect was revealed. This patient and two similar ones which we have observed in the past three years lead us to think that much of the idiopathic steatorrhea, often mistakenly classified as sprue or nontropical sprue, and which is stubbornly resistant to treatment with liver or pteroylglutamates may be primary gastrointestinal disease with conditioned anemias.

#### SUMMARY

Eleven cases treated with vitamin B<sub>12</sub> have been presented. Eight patients with pernicious anemia in relapse responded hematologically. Two patients with mild neurologic involvement were relieved by therapy with B<sub>12</sub> alone.

Consideration of the quantities of the crystalline vitamin required to promote maximal erythropoiesis in pernicious anemia indicates that less than about 0.75

$\mu\text{g}$  daily in doses at intervals of several days will not suffice to establish and maintain blood values as high as does adequate treatment with liver extract. Parenteral daily doses of  $10 \mu\text{g}$  promoted good erythropoiesis in one patient, although it appears that the maximum rate of hemopoiesis may require the initial average daily dose of approximately  $30 \mu\text{g}$ .

The reticulocyte count is an unreliable quantitative criterion of activity or adequacy of therapy.

It is suggested that hemopoietic factors in addition to PGA and  $\text{B}_{12}$  may be required by some patients to obtain maximal erythrocyte levels.

Vitamin  $\text{B}_{12}$ , as well as PGA, effects a reduction in the fecal urobilinogen output of patients with pernicious anemia. The significance of this finding is discussed.

No change in urinary excretion of pteroylglutamate or of porphyrin was detected in patients treated with vitamin  $\text{B}_{12}$ .

#### REFERENCES

- <sup>1</sup> RICKES E L, BRINK N G, KONIUSZY F R, WOOD, T R AND FOLKERS K. Crystalline vitamin  $\text{B}_{12}$ . *Science* 107 396 1948
- <sup>2</sup> SMITH, E. L. Purification of anti pernicious anemia factors from liver. *Nature* 161 638, 1948
- <sup>3</sup> WEST, R. Activity of vitamin  $\text{B}_{12}$  in Addisonian pernicious anemia. *Science* 107 398, 1948
- <sup>4</sup> SEBORG M S. Activity of vitamin  $\text{B}_{12}$  for the growth of *Lactobacillus lactis*. *Science* 107 397 1948
- <sup>5</sup> RICKES E L, BRINK N G, KONIUSZY, F R, WOOD T R. AND FOLKERS K. Vitamin  $\text{B}_{12}$ : a cobalt complex. *Science* 108 134 1948
- <sup>6</sup> SMITH E L. Presence of cobalt in the anti pernicious anaemia factor. *Nature* 162 144, 1948
- <sup>7</sup> — AND PARKER, L. F. J. Purification of anti pernicious anaemia factor. *Proc Biochem Soc*, May 29, 1948
- <sup>8</sup> RICKES, E L., BRINK, N G, KONIUSZY F R., WOOD, T R., AND FOLKERS, K. Comparative data on vitamin  $\text{B}_{12}$  from liver and from a new source, *Streptomyces griseus*. *Science* 108 634 1948
- <sup>9</sup> BERK, L., DENNY-BROWN D, FINLAND M., AND CASTLE W B. Effectiveness of vitamin  $\text{B}_{12}$  in combined system disease. *New England J Med* 239 328, 1948
- <sup>10</sup> SPIES T D, STONE R. E. AND ARAMBURU T. Observations on the antianemic properties of vitamin  $\text{B}_{12}$ . *South M. J* 41 522, 1948
- <sup>11</sup> — LOPEZ, G G, MILANES F, TOCA R. L. AND CULVER B. Observations on the hemopoietic response of persons with tropical sprue to vitamin  $\text{B}_{12}$ . *South M. J* 41 523, 1948
- <sup>12</sup> — AND SUAREZ R M. Response of tropical sprue to vitamin  $\text{B}_{12}$ . *Blood* 3 1213 1948
- <sup>13</sup> JONES, E. TILLMAN C AND DARBY W J. Observations on relapses in pernicious anemia. *Ann Int Med* 30 374 1949
- <sup>14</sup> DARBY W J, JONES E AND JOHNSON, H C. Effect of synthetic lactobacillus casei factor in treatment of sprue. *J A M A* 130 780 1946
- <sup>15</sup> WATSON, C J. Studies of urobilinogen I. An improved method for the quantitative estimation of urobilinogen in urine and feces. *Am J Clin Path* 6 458, 1936
- <sup>16</sup> STEINKAMP R. C, SHUKERS C F, TOTTER, J R AND DAY P L. The excretion of porphyrin by pernicious anemia patients treated with pteroylglutamic acid. *Fed Proc* 6 295 1947
- <sup>17</sup> DARBY W J. The physiological effects of the pteroylglutamates in man—with particular reference to pteroylglutamic acid (PGA). *Vitamins and Hormones* 5 119 1947
- <sup>18</sup> — The treatment of nutritional anemias with folic acid. *Symposia on Nutrition Vol I, Nutritional Anemia* p 61 Cincinnati Ohio Robert Gould Research Foundation Inc 1947
- <sup>19</sup> FROMMETER W B, JR AND SPIES T D. Relative clinical and hematologic effects of concentrated liver extract, synthetic folic acid and synthetic 5 methyl uracil in the treatment of macrocytic anemias in relapse. *Am J M. Sc* 213 135, 1947
- <sup>20</sup> ISAACS R AND FRIEDMAN A. Standards for maximum reticulocyte percentage after intramuscular liver therapy in pernicious anemia. *Am J M Sc.* 196 718 1938

- <sup>21</sup> BETHELL F H METERS, M C AND NELIGH, R. B Vitamin B<sub>12</sub> in pernicious anemia and puerperal macrocytic anemia J Lab & Clin Med 33 1477, 1948
- <sup>22</sup> CLARK, G W A survey of the treatment of pernicious anemia in relapse Am J M. Sc. 216 71 1948
- <sup>23</sup> FARQUHARSON R F BORRICK, H AND GOULDING A M Pigment metabolism and destruction of blood in Addison's (pernicious) anemia Arch Int Med 48 1156 1931
- <sup>24</sup> SINGER K KING J C, AND ROBIN, S The life span of the megalocyte and the hemolytic syndrome of pernicious anemia J Lab & Clin Med 33 1068 1948
- <sup>25</sup> LONDON I M., WEST R SHEMIN D AND RITTENBERG D On the origin of stercobilin in humans Fed Proc 7 169 1948
- <sup>26</sup> HALL, B E., AND CAMPBELL, D C. Vitamin B<sub>12</sub> therapy in pernicious anemia I An effect on hematopoietic system Preliminary report Proc. Staff Meet. Mayo Clin 23 584, 1948

# COMPARISON OF VITAMIN B<sub>12</sub> FROM LIVER AND FROM *STREPTOMYCES GRISEUS* IN THE TREATMENT OF PERNICIOUS ANEMIA

By LOWELL A. ERF, M.D., AND BRUCE WIMER, M.D.

THIS communication describes the results of treatment of three cases of pernicious anemia in relapse, one with vitamin B<sub>12</sub>\* derived from liver and two with vitamin B<sub>12</sub>† derived from *Streptomyces griseus*, the latter preparation has been used also in 5 cases of pernicious anemia in remission.

Vitamin B<sub>12</sub>, a crystalline material isolated from liver, has been shown to be a potent anti-pernicious anemia substance. In 17 reported cases of pernicious anemia in relapse (table 1), all have shown definite hematologic responses when vitamin B<sub>12</sub> was administered in microgram doses. There was a prompt increase in circulating reticulocytes in those cases where the initial erythrocyte count was below 2,000,000. In all 17 cases there was a rise in hemoglobin levels and the erythrocyte counts, the levels usually approached normal in six to eight weeks if the total dosage was adequate. The bone marrow regenerated promptly (48-72 hours) from a rubriblastic<sup>7</sup> (megaloblastic) hyperplasia to a rubricytic (normoblastic) hyperplasia.<sup>6</sup>

The response of neurologic complications was followed in 13 of the 17 cases of pernicious anemia.<sup>1, 4, 12</sup> The initial observations indicate that vitamin B<sub>12</sub> is effective, as is liver extract, in producing at least a partial remission of the neurologic manifestations. There was virtually a complete remission in one case which was treated shortly after the onset of neurologic complaints.<sup>1</sup> In the other cases paresthesia, ataxia and Romberg's phenomenon responded reasonably well while the loss of vibration sense and position sense were relatively more resistant to treatment.<sup>4</sup>

A vitamin B<sub>12</sub>-like substance has recently been isolated from cultures of *Streptomyces griseus*.<sup>8</sup> The crystals isolated from this new source have physical and chemical properties very similar to crystalline vitamin B<sub>12</sub> from liver and the two substances have almost the same growth promoting potency for *Lactobacillus lactis* Dorner, and West<sup>8</sup> has found that the clinical response in pernicious anemia to the new substance parallels that produced by crystalline vitamin B<sub>12</sub>.<sup>16</sup>

## CASE REPORTS

### *Vitamin B<sub>12</sub> from Liver: The Treatment of One Case of Pernicious Anemia in Relapse*

**History.** Mrs. A. O. B., a 64 year old white woman of Irish descent was admitted to Jefferson Hospital September 24, 1948 because of progressive weakness starting July, 1948. Anorexia became a major

From the Charlotte Drake Cardeza Foundation and Department of Medicine, Jefferson Medical College and Hospital, Philadelphia, Pa.

\* Vitamin B<sub>12</sub> derived from liver supplied through the courtesy of Dr. A. Gibson, Merck and Co. Rahway, New Jersey.

† Vitamin B<sub>12</sub> derived from *Streptomyces griseus* supplied through the courtesy of Dr. Charles Mann, E. R. Squibb & Sons, New York City.



complaint. Her eyes tired easily during the week before admission and two days previous to admission tinnitus would occur when she lay down. She also noted numbness and tingling of the fingers. One day prior to admission she found that she had to use her arms to support herself when she stood up. Her weakness bordered on collapse and she was sent to the hospital.

**Past History.** The patient's general health had been good until 1932, when she was found to have hypertension. She had epistaxis at that time and again on two subsequent occasions. In July 1946 and in April 1948 she had two severe vascular accidents with hemiplegia. Recovery was good but not complete in that the patient still complained of uneven gait and weakness of the right upper extremity at the time of admission. She had lost 23 pounds of weight during 1947 and 1948.

**Physical Examination.** The patient was a white haired blue-eyed woman of short medium build. There was marked pallor of the skin, conjunctivae and mucous membranes. An herpetic lesion was seen on the left upper lip. The lingual papillae were atrophic. The blood pressure was 120/70. The neurologist reported the following findings: Grade II arteriosclerosis of the retinal vessels; slight weakness of the right upper and lower extremities as compared with the left; reflexes hyperactive bilaterally; plantar reflex weakly extensor on the right; vibration sense of the legs and ankles slightly decreased; impairment of position sense of both large toes; gait unsteady; swaying in performance of Romberg's

TABLE 1—Treatment of Pernicious Anemia with Vitamin B<sub>12</sub> from Liver

First Author Only	Number of Cases of Pernicious Anemia	Dosage of Vitamin B <sub>12</sub>
West <sup>12</sup>	3	Single doses of 3, 6, and 150 $\mu$ g respectively
Spies <sup>11</sup>	2	Single doses of 6 $\mu$ g and 15 $\mu$ g
Berk <sup>1</sup>	1	5 $\mu$ g daily for 8 days and 5 $\mu$ g 3 times weekly from 16th to 60th day
Hall <sup>6</sup>	11	Total of 40 to 325 $\mu$ g during in intervals of 30 to 50 days
	—	
	17	

test and single foot standing. The patient was responsive but there was some confusion and memory impairment. The neurologist's impression was that there were signs of combined system disease attributed primarily to arteriosclerosis and secondarily to pernicious anemia.

**Laboratory Findings.** Hemoglobin 35.6 per cent (5.4 Gm). Erythrocytes 1,580,000. Leukocytes 4,400. Platelets 72,000. Differential: segmented polys, 77 per cent; medium and small lymphocytes 17 per cent; monocytes 6 per cent. Smear of peripheral blood—typical macrocytic anemia with anisocytosis and poikilocytosis. Hematocrit, 17 per cent. Mean corpuscular volume, 107. Bone marrow rubriblastic (megaloblastic) (see fig. 1). Urine concentration test, 1:006, 1:010, 1:010. Gastric analysis achlorhydria (with histamine). Gastroscopic examination: severe degree of atrophy of the stomach mucosa. Fluoroscopic examination of the chest: healed tuberculous lesion of the left apex. Heart had an aortic configuration and was hypertrophied 10 to 15 per cent above normal. Electrocardiogram: left axis deviation. Intravenous pyelogram: ptosis of the kidneys bilaterally, in the upright position.

**Diagnosis.** (1) Pernicious anemia in relapse. (2) Combined system disease secondary to arteriosclerosis and pernicious anemia. (3) Cerebral arteriosclerosis. (4) Arteriosclerotic cardiovascular renal disease.

**Treatment.** The patient was given 25  $\mu$ g of vitamin B<sub>12</sub> from liver on October 4 and a second dose of 25  $\mu$ g on October 5, 1948.

**Course.** The hematologic changes are shown in table 2. The reticulocytes rose the second day after injection and reached a maximum of 33.8 per cent on the sixth day. The ascent of the erythrocyte count and hemoglobin level started at the end of the first week and a peak of 3,900,000 (erythrocyte count) and 83 per cent or 12.8 Gm (hemoglobin) was reached December 29, 1948, eighty-six days after treatment was instituted. The counts then started to decline, reaching 3,200,000 (erythrocyte count) and

68.5 percent or 10.5 Gm (hemoglobin) on January 16, 1949, the 114th day. The bone marrow had become rubricytic by the sixth day after injections of vitamin B<sub>12</sub> (fig. 2).

Improvement in the patient's strength and appetite started within three or four days after the injections. Mentally she became more alert. Paresthesias did not recur and the patient was able to walk without assistance although her gait was somewhat cautious and onsteady. Following her discharge from the hospital October 18, 1948, the patient's symptomatic improvement paralleled the rise in blood count. However, when the erythrocyte count exceeded 3,000,000 the elevation of blood pressure returned, varying between 160/90 and 180/100. A re-evaluation by the neurologist January 24, 1949, reported the same findings as previously except for the presence of normal position sense. At this time it was thought that positive neurologic findings were due to the previous strokes. Even at the time that the blood levels started to fall (January 24, 1949) the patient stated she felt well.

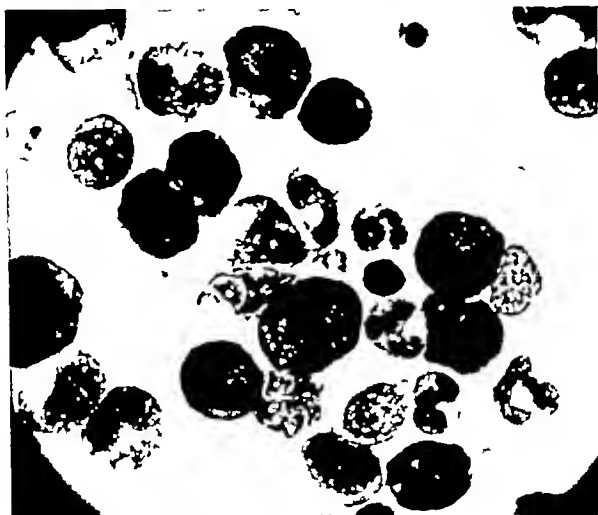


FIG. 1.—RUBRICYTIC HYPERPLASIA

Sternal marrow obtained from Mrs. A. O. B. on 9-31-48 before the administration of vitamin B<sub>12</sub> from liver.

### *Vitamin B<sub>12</sub> from Streptomyces griseus: The Treatment of Two Cases of Pernicious Anemia in Relapse*

**Case I History.** Mr. A. C., a 67-year-old Italian laborer, had been treated for pernicious anemia in the Hematology Out Patient Department since 1939. The anemia was adequately controlled with liver extract, but in September 1948 he stopped coming to clinic because he thought he was cured.

In November 1948 the patient noticed the onset of progressive weakness and in a month's time he was too incapacitated to continue work. He began to experience intermittent nausea and vomiting and epigastric distress after eating. His appetite declined and finally became so poor that he ate virtually nothing during the three days before he was admitted to the Jefferson Hospital January 7, 1949.

**Physical Examination.** The patient was a well-nourished elderly Italian with pronounced pallor of the skin and mucous membranes. Sclerae and buccal mucosae were faintly icteric. The tongue was pale, smooth and glistening. The heart was enlarged and the rhythm was irregular because of frequent extrasystoles. A systolic murmur was heard over the entire precordium, loudest over the aortic area. The liver was enlarged 3-4 cm. below the costal margin on deep inspiration and the tip of the spleen could be felt. The blood pressure was 120/80. The neurologist could find no abnormal neurologic changes.

*Laboratory Findings* Hemoglobin 40 per cent (6.16 Gm) Erythrocytes 1,610,000 Reticulocytes 1.1 Leukocytes 4,450 Differential segmented polys 57 per cent medium and small lymphocytes, 38 per cent monocytes 8 per cent normoblasts, 1 per cent smear of peripheral blood revealed macrocytosis anisocytosis, poikilocytosis and rubricytosis Bone marrow rubriclastic and prnrubricytic (see fig 3)

TABLE 2.—Mrs A O B (Admitted 9-24-48) Hematologic Response After Administration of Vitamin B<sub>12</sub> from Liver

Date	Day (after 1st dose)	Hemoglobin		RBC $\times 10^9$	Reticulo-cytes	WBC $\times 10^3$	Remarks
		%	Gm				
9-29-48		35.6	5.4	1.58	—	4.4	
10-1-48		—	—	—	1.3	—	9-31-48 Bone marrow rubriclastic (fig 1)
10-2-48		35.6	5.4	1.840	1.8	3.0	
10-4-48	1	—	—	—	—	—	Vitamin B <sub>12</sub> 25 $\mu$ g
10-5-48	1	—	—	—	—	—	Vitamin B <sub>1</sub> 25 $\mu$ g
10-6-48	2	—	—	—	7.4	—	
10-7-48	3	—	—	—	9.2	—	
10-8-48	4	37.2	5.7	1.5	23.6	—	Early improvement of appetite strength and mental response
10-9-48	5	—	—	—	21.0	—	
10-10-48	6	—	—	—	33.8	—	
10-11-48	7	45.5	7.0	1.78	26.5	—	10-11-48 Bone marrow rubricytic and metarubricytic (fig 2)
10-12-48	8	—	—	—	15.5	—	
10-13-48	9	—	—	—	16.7	—	
10-14-48	10	—	—	—	15.3	—	
10-15-48	11	—	—	—	13.2	—	
10-16-48	12	—	—	—	9.4	—	Discharged from hospital 10-18-48
10-27-48	23	72	10.9	2.61	1.2	2.7	
11-3-48	30	73.6	11.3	3.88	—	—	Appetite good Steady increase in strength
11-17-48	44	75.6	11.6	3.47	—	—	
12-1-48	58	76	11.7	3.63	—	—	
12-29-48	86	83.1	12.8	3.9	—	—	Felt better than any time during past 20 years
1-19-49	107	73.2	11.2	3.85	1.0	3.0	
1-24-49	112	75.2	11.6	3.48	0.71	5.0	Still felt well
1-26-49	114	68.5	10.5	3.22	0.6	—	

\* Based on 15.6 Gm as 100 per cent.

Serum bilirubin 1.3 mg Urea clearance 68 per cent Roentgenogram of chest a boot shaped cardiac silhouette with enlargement to the left The lung fields were clear Gastro-intestinal series negative Gastroscopic examination benign nonbleeding polyp of the antrum and diffuse atrophy of gastric mucosae Electrocardiogram occasional premature auricular contractions and left axis deviation

*Diagnosis* (1) Pernicious anemia in relapse (2) Benign polyp of antrum of stomach

*Treatment* The patient was given 32  $\mu$ g of vitamin B<sub>12</sub> from *Streptomyces griseus* on January 7 1949.

*Course* The hemoglobin and reticulocyte levels are shown in table 3 The reticulocytes started to rise between 48-72 hours and reached a maximum of 40 per cent the fifth and sixth days after the injection of vitamin B<sub>12</sub> from *Streptomyces griseus* The bone marrow aspiration on January 13 the sixth postinjection

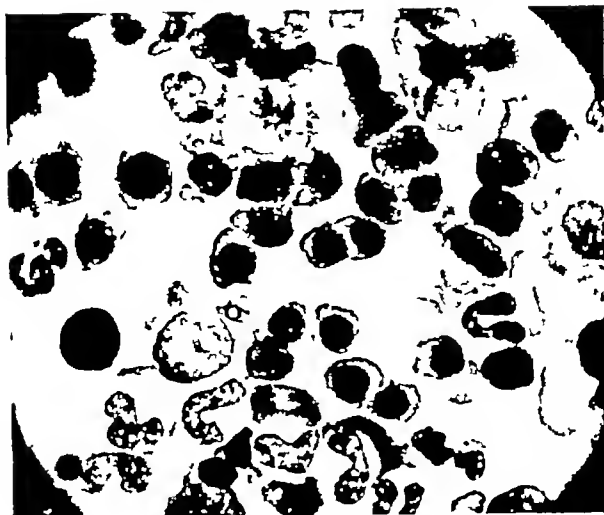


FIG. 2.—RUBRICYTIC AND METARUBRICYTIC HYPERPLASIA

Sternal marrow obtained from Mrs. A. O. B. on 10-11-48 six days after administration of 50  $\mu$ g of vitamin B<sub>12</sub> from liver

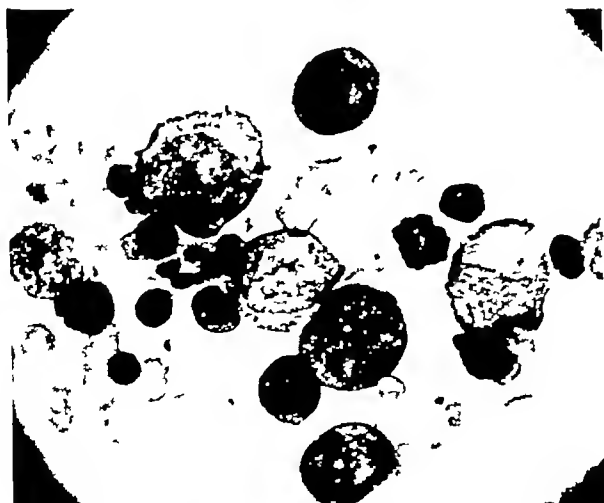


FIG. 3.—RUBRIBLASTIC AND PRORUBRICYTIC HYPERPLASIA

Sternal marrow obtained on 1-7-49 from Mr. A. C. before the administration of vitamin B<sub>12</sub> from *Streptomyces griseus*

tion day showed numerous rubricytes and metarubricytes (Fig. 4). The erythrocyte count had risen to 3,190,000 and the hemoglobin level had risen to 55.4 per cent or 8.54 Gm. by the nineteenth day. Symp-

tomatically the patient improved rapidly. He felt notably stronger and his appetite became normal before he was discharged January 14, 1949. The patient returned to work January 24 and he continued to have a good appetite and an increase in endurance during the nineteen day period of observation.

TABLE 3—Mr. A. C. (Admitted 1-7-49) Hematologic Response After Administration of Vitamin B<sub>12</sub> from *Streptomyces griseus*

Date	Day (after 1st dose)	Hemoglobin		RBC $\times 10^6$	Reticu- locytes	WBC $\times 10^3$	Remarks
		%	Gm.				
1-7-49	0	40	6.16	1.61	1.1	4.45	1-7-49 Bone Marrow Rubn blastic and protorubricytic. (fig. 3) 32 $\mu$ g Vitamin B <sub>12</sub> i.m.
1-8-49	1	—	—	—	2.2	—	
1-10-49	3	31.6	4.88	1.75	16.2	—	
1-11-49	4	—	—	—	40.0	—	Improvement in strength and ap- petite
1-12-49	5	—	—	—	40.0	—	
1-13-49	6	—	—	—	32.8	—	1-13-49 Bone Marrow Rubn cytic (fig. 4)
1-14-49	7	—	—	—	17.5	—	Discharged 1-14-49
1-19-49	12	43.0	6.6	2.59	19.8	7.8	Returned to work 1-24-49
1-26-49	19	55.4	8.54	3.14	4.5	4.5	

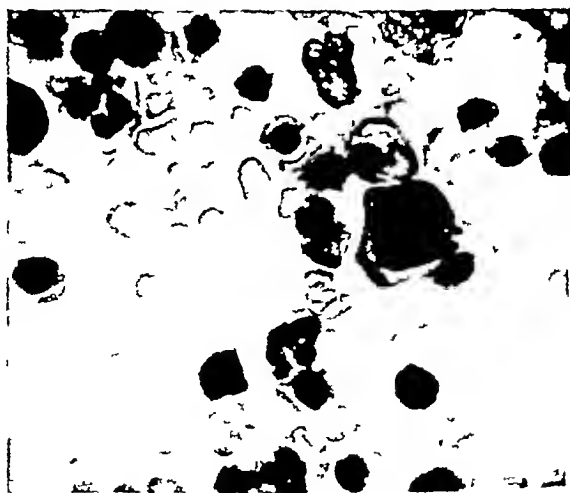


FIG. 4—RUBRICYTIC HYPERPLASIA

Sternal marrow obtained on 1-13-49 from Mr. A. C., six days after the administration of 32  $\mu$ g of vitamin B<sub>12</sub> from *Streptomyces griseus*.

**Case II History.** Mrs. K. R., a 47-year-old secretary of Irish descent, was first seen January 12, 1949 because of pallor, weakness and staggering. The patient noticed onset of progressive weakness early in November 1948. Two weeks later she found it necessary to stop work because of exhaustion. Although she was able to perform minimal household tasks her clinical course was downhill. At the end of Decem-

ber she developed staggering and her appetite began to wane. In January, her legs became increasingly edematous. She attributed these symptoms to a rundown condition but the exhaustion and pallor in her lips finally became so striking she had to consult a physician.

**Past History.** About ten years previously the patient had an episode of weakness which necessitated six months period of rest at home. Her physician advised a diet high in liver, meat and vegetables. She was given a few injections the nature of which she did not know. The symptoms gradually subsided and although she never felt well she was able to work the succeeding nine years until December 1947 when weakness again became incapacitating. She was placed on the same diet and given an oral tonic. Symptoms subsided to the extent that she was able to start work in April 1948 and continue until the onset of the present episode in November 1948.

**Physical Examination.** The patient was a white haired, blue-eyed white woman with extreme pallor. She had to be supported by two people when she walked because of weakness and staggering gait. There was pronounced mental dullness, confusion and memory loss. Her tongue was pale with smooth glistening edges. There were scattered areas of vitiligo and brownish pigmentation of the skin over the shoulders.

TABLE 4.—Mrs. K. R. Hematologic Response After Administration of Vitamin B<sub>12</sub> from *Streptomyces griseus*

Date	Day (after 1st dose)	Hemoglobin		RBC $\times 10^6$	Reticu- lyocytes	WBC $\times 10^3$	Remarks
		%	Gm.				
1-12-49	0	22	3.25	1.02	1.6	3.5	1-12-49 Bone Marrow Rubri- blastic (fig 5) Vitamin B <sub>12</sub> 32 $\mu$ g i.m.
1-14-49	2	22	3.25	1.06	2.5	3.7	1-14-49 Bone Marrow Prorubri- cytic and rubricytic (fig 6) 41 hours after injection
1-15-49	3	26	4.0	1.05	20.3	4.0	Increased appetite and strength
1-17-49	5	30	4.6	1.2	31.2	4.0	Able to walk without assist- ance
1-19-49	7	28	4.25	1.25	16.7	4.2	
1-21-49	9	30	4.6	1.65	15.7	5.6	Vitamin B <sub>12</sub> 32 $\mu$ g
1-22-49	10	33	5.0	1.97	15.0	7.4	
1-24-49	12	33	5.0	1.9	13.5	5.7	
1-26-49	14	35	5.5	1.92	9.2	6.2	Toogoe normal
1-28-49	16	38	6.0	1.94	5.2	6.4	Color good Edema gone

and at the neckline. The heart sounds were rapid and weak and there were fine rales at both bases. The skin was dry and waxy and there was pronounced edema of the hands, legs and ankles. Neurologic findings included the following: Pronounced weakness of hand grip (without atrophy), hyperactive biceps, triceps and quadriceps reflexes, a positive Hoffmann's sign more pronounced on the left, hyperesthesia about the ankles, impaired large toe position sense, positive Babinski's sign bilaterally, absence of vibration sense up to the iliac crests, where it was impaired, pronounced ataxia and positive Romberg's sign.

**Laboratory Findings.** Hemoglobin 22 per cent (3.25 Gm). Erythrocytes, 1,020,000. Reticulocytes, 1.6 per cent. Leukocytes, 3,500. Differential polys 45 per cent, eosin 3 per cent, myelocytes 1 per cent, lymphocytes 51 per cent. Normoblasts, 17 per 100 WBC, smear of peripheral blood was characteristic of pernicious anemia showing macrocytosis, anisocytosis, poikilocytosis and rubricytosis. Bone marrow rubriblastic (fig 5).

**Diagnosis.** (1) Pernicious anemia in relapse. (2) Subacute combined degeneration.

**Treatment.** An injection of 32  $\mu$ g of vitamin B<sub>12</sub> from *Streptomyces griseus* was given on January 12, 1949. The dose was repeated January 21, 1949 because of the severity of neurologic symptoms.

**Course.** The hemoglobin and reticulocyte levels are shown on table 4. There was a prompt rise of reticulocytes starting 48-72 hours after the first injection and reaching a maximum about the fifth day. The bone marrow 41 hours later showed maturation of rubriblasts toward prorubricytes (fig 6). The erythrocyte count started to rise significantly the fifth day and reached 1,940,000 by the sixteenth day.

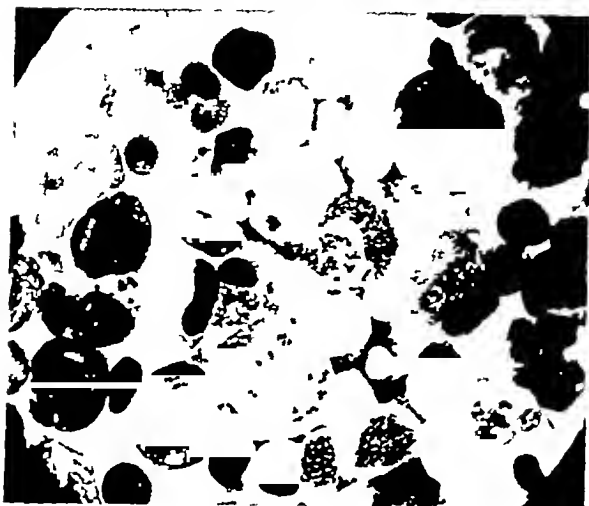


FIG 5—RUBRIBLASTIC HYPERPLASIA

Sternal marrow obtained on 1-12-49 from Mrs. K. R. before the administration of vitamin B<sub>12</sub> from *Streptomyces griseus*

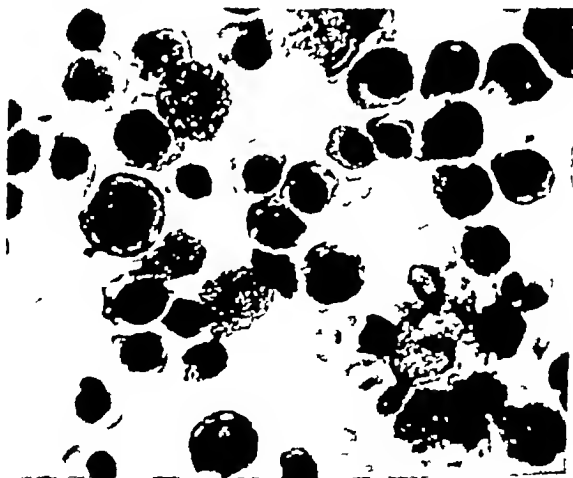


FIG 6—PRORUBRICYTIC AND RUBRICYTIC HYPERPLASIA

Sternal marrow obtained on 1-14-49 from Mrs. K. R., 41 hours after the administration of 32 µg of vitamin B<sub>12</sub> from *Streptomyces griseus*

Weakness and anorexia started to disappear within four days. By the sixth day the patient was able to resume physical activity without help. Increased color became evident about the seventh day. The edema of the hands and feet gradually subsided. Mentally, the patient was still retarded on the fifth day but was

able to give fragments of the past history previously denied. By the ninth day she was almost normally responsive and was able to give particulars of her past history. The neurologic improvement is shown in table 5. Neurologically there was steady improvement of ataxia and performance of Romberg's test the first week. On the sixteenth day the gait was only slightly ataxic. There was a slower improvement in the other neurologic findings.

TABLE 5—Mrs. A. R. Response of Neurologic Manifestations to Vitamin B<sub>12</sub> from *Streptomyces griseus*

Date	1-12-49	1-14-49	1-21-49	1-24-49	1-26-49	1-28-49
Day after 1st dose	0	5	9	12	14	16
Day after 2nd dose	—	—	0	3	5	7
Mental response	Pronounced dullness confusion	Slow	Better	Normal	Normal	Normal
Ataxia	4	2	1	1	1	Slight
Romberg's sign	4	2	Swaying	Swaying	Slight swaying	Same
Vibration sense						
Anterior superior iliac spine	Right—2 Left—3	Right—2 Left—3	Right—2 Left—3	Right—1 Left—2	Right—normal Left—2	Right—normal Left—2
Upper part of tibia	Absent	Absent	Absent	Right—Faint Left—Absent	Right—Faint Left—Absent	Right—Faint Left—Absent
Ankle	Absent	Absent	Absent	Absent	Absent	Absent
Position sense—toe	Absent	Absent	Impaired	Impaired	Impaired	Impaired
Heel knee test	Right—4 Left—4	Right—3 Left—3	Right—2 Left—3	Right—1 Left—2	Slight unsteadiness	Slight unsteadiness
Tendon reflexes						
Biceps and triceps	4	4	4	3	2	2
Quadriceps	4	4	4	3	2	2
Hoffman's sign	Right—2 Left—4	Right—2 Left—3	Right—1 Left—3	Right—2 Left—3	Right—2 Left—3	Right—2 Left—2
Babinski's sign	Right—3 Left—3	Right—3 Left—3	Right—3 Left—3	Right—2 Left—2	Right—2 Left—2	Right—1 Left—1

### Vitamin B<sub>12</sub> from *Streptomyces Griseus* The Treatment of Five Cases of Pernicious Anemia in Remission

A single dose of 32 µg of vitamin B<sub>12</sub> from *Streptomyces griseus* was given intramuscularly to each of five patients with pernicious anemia who had been receiving liver extract regularly for periods varying from six months to ten years. All but one of these patients had associated diseases: arteriosclerosis and cystitis (case 3), diabetes mellitus (case 4), hypothyroidism (case 5), and latent lues (case 7). Four patients (cases 3, 5, 6, 7) appeared rather resistant to treatment with liver extract in that their erythrocyte levels remained below optimum or they had complained frequently of weakness and/or poor appetite.

The hematologic responses following injection of vitamin B<sub>12</sub> from *Streptomyces griseus* are shown in table 6. There was a slight reticulocyte increase (1.8–4.9 per cent) in only one patient (case 7) whose initial erythrocyte count was 3,000,000. In one patient (case 6) there was a significant rise in erythrocyte count.



TABLE 6—Hematologic Response to Vitamin B<sub>12</sub> from *Streptomyces griseus* Five Patients with Pernicious Anemia in Remission

Date	Day (after 1st dose)	Hemoglobin		RBC $\times 10^6$	Reticu- locytes	WBC $\times 10^3$	Level of RBC $\times 10^6$ during 2 months previous to injection		
		%	Gm.				Range	Ave.	
(Case 3—F P)								3.4 to 4.4	3.8
12-22-48	0	82.3	12.8	3.82	0.5	6.0			
12-24-48	2	—	—	—	1.0	—			
12-27-48	5	—	—	—	1.8	—			
12-29-48	7	79.2	12.2	3.45	0.7	—			
12-31-48	9	—	—	—	1.2	—			
1-10-49	10	—	—	—	1.1	—			
1-12-49	21	78.7	12.1	3.96	—	5.6			
1-19-49	28	79.6	12.2	3.77	2.5	6.5			
1-26-49	35	87.1	13.4	—	—	—			
(Case 4—M. S.)								3.9 to 4.2	4.05
12-22-48	0	81.5	12.5	4.28	1.1	6.2			
12-31-48	9	—	—	—	1.3	—			
1-5-49	14	73.2	11.3	3.68	1.8	5.6			
1-12-49	21	76.5	11.6	4.0	3.2	5.9			
1-19-49	28	80.7	12.4	3.86	1.5	5.2			
1-26-49	35								
(Case 5—A. N.)								3.8 to 4.5	4.2
12-29-48	0	84.7	13.0	4.13	0.3	—			
12-31-48	2	—	—	—	0.6	—			
1-3-49	5	—	—	—	1.4	—			
1-5-49	7	76.8	11.8	3.76	0.3	5.6			
1-7-49	9	—	—	—	0.9	—			
1-10-49	12	—	—	—	0.5	—			
1-12-49	14	75.6	11.6	4.05	0.7	—			
1-19-49	21	80.0	12.3	3.74	0.7	5.7			
1-26-49	28								
(Case 6—M. B.)								3.0 to 3.7	3.3
12-22-48	0	68.1	10.4	3.37	0.4	5.5			
12-24-48	2	—	—	—	1.0	—			
12-27-48	5	—	—	—	0.6	—			
12-29-48	7	77.2	11.9	3.51	0.6	—			
12-31-48	9	—	—	—	0.5	—			
1-5-49	14	77.2	11.9	4.02	1.1	4.0			
1-12-49	21	70.8	10.9	3.83	0.9	—			
1-19-49	28	72.0	11.1	4.1	0.4	4.6			
1-26-49	35	79.2	12.2	4.17	—	—			
(Case 7—V. S.)								2.7 to 3.2	2.9
1-12-49	0	64.9	10.0	3.2	1.8	4.2			
1-14-49	2	—	—	—	1.9	—			
1-17-49	5	—	—	—	4.9	—			
1-26-49	14	73.2	11.3	3.3	1.2	3.7			

The clinical response to treatment is indicated in table 7. Four of the five reported subjective improvement (cases 3, 5, 6, 7) mainly better appetite, increased strength and more endurance. In two instances there was significant weight gain (case 3 7½ lbs, case 6 13 lbs).

### DISCUSSION

Shorb<sup>10</sup> found by microbiologic assay that vitamin B<sub>12</sub> has 11,000 times more growth promoting potency for *Lactobacillus lactis* Dorner than a standard concentrated liver extract and estimated by this method that 1 µg of vitamin B<sub>12</sub> is

TABLE 7—Clinical Response of Five Patients with Pernicious Anemia in Remission Treated with Vitamin B<sub>12</sub> from *Streptomyces griseus*

Case	Sex age color	Diagnosis made	Treatment (dosage of liver extract)	Associated diagnoses	Results of injection of 32 µg	Period observed
						weeks
3 F P	M 61 W	1945	15 U 2 x weekly	Coronary arteriosclerosis P V D—legs (Arteriosclerosis) Chronic cystitis	Increased appetite and strength Weight gain 7½ lbs	5
4 M S	F 53 W	1939	15 U q 1-3 weeks	Diabetes mellitus Arthritis Bursitis	No improvement. (Diabetes difficult to control because of poor cooperation)	5
5 A. N	F 56 W	1946	15 U q 1-2 weeks	Hypothyroidism	Increased strength Appetite still poor	3
6 M. B	F 44 W	1948 (June 28)	15 U q week	None	Increased strength endurance and appetite. Weight gain—12½ lbs Rise in erythrocyte count	5
7 V S	F 45 C	1942	15 U q 2 weeks	Latent lues (Treated in 1942)	Increased strength endurance and appetite	2

approximately equivalent to 1 U S P unit of liver extract. Clinical studies, on cases of pernicious anemia, also imply that this ratio is approximately the same for the anti-pernicious anemia activity of the two substances. Thus, for example, in the first case reported in this paper, the dosage of 50 µg of vitamin B<sub>12</sub> produced a rise of circulating erythrocytes from 1,600,000 to 3,900,000 per cu mm. From previous computations<sup>13</sup> a similar rise of 2,300,000 erythrocytes per cu mm should have been obtained by the daily administration of 1 to 2 units of liver extract for a four and one-half week period (total dosage between 32 and 64 units). In general it appears that a satisfactory remission can be produced by a dosage of 50-100 µg of vitamin B<sub>12</sub>, whereas the number of U S P units of liver extract necessary to pro-

duce a remission is about 56-102.<sup>13</sup> The relative accuracy of this correlation between the clinical response to vitamin B<sub>12</sub> and its LLD potency emphasizes the importance of the microbiologic assay method.

In treating the first case (Mrs. A. O. B.) a dose of 50 µg of vitamin B<sub>1</sub> from liver was given during the first two days and further treatment was withheld during a 114 day period of observation to determine the type and duration of response to this method of administration. The hematologic remission was probably suboptimal because the erythrocyte count did not reach the desired level. There was, however, complete remission of all symptoms attributable to pernicious anemia. The rise in red cell levels lasted about 86 days, at the end of which time the erythrocyte count and hemoglobin levels started to descend. A suboptimal response following a single initial dose would depend upon either an inadequate dose or an inability of the patient to store amounts in excess of the immediate need at the time of injection.

The hematologic improvement of 2 cases of pernicious anemia in relapse (Mr. A. C. and Mrs. K. R.) treated with vitamin B<sub>12</sub> derived from *Streptomyces griseus* is similar to that obtained by others who gave vitamin B<sub>12</sub> from liver and used dosages of the same magnitude. These two patients had an early reticulocyte rise which was followed by a steady elevation of the erythrocyte level. The clinical symptoms of weakness, exhaustion, and anorexia disappeared rapidly and the neurologic improvement in one case (Mrs. K. R.) was definite from a functional standpoint although the tendency for some of the physical signs, such as pathologic reflexes, loss of vibration and position sense, to disappear was slow during a two week period of observation. These changes are not unlike the results obtained in the treatment of similar cases with vitamin B<sub>12</sub>.<sup>1-4</sup> These initial results make it appear that from the clinical standpoint both vitamin B<sub>12</sub> from liver and vitamin B<sub>12</sub> from *Streptomyces griseus* are either closely similar or identical in their physiologic action.

The administration of vitamin B<sub>12</sub> from *Streptomyces griseus* to 5 patients with pernicious anemia in remission resulted in minor subjective and objective improvements in 4 of the 5 cases. In one patient who had been receiving 15 U.S.P. units liver extract every week, there was a significant rise in the erythrocyte count (3,300,000 to 4,000,000 cu. mm.) following the injection of vitamin B<sub>12</sub> from *Streptomyces griseus*. It is probable that an equivalent dosage (i.e., about 32 units) of liver extract would have produced a similar result.

All evidence available at present indicates that vitamin B<sub>12</sub> is the active anti-pernicious anemia factor in liver extract. Its reaction in cases of pernicious anemia is in every way comparable to that of liver extract, differing only in that it produces an earlier reticulocyte rise and a reticulocyte peak on the fifth day compared to the seventh day with liver extract. Vitamin B<sub>12</sub> has the advantage that it is nonirritating when injected intramuscularly. Berk et al.<sup>1</sup> reported a case allergic to both beef and pork liver extract but who developed no reaction when vitamin B<sub>12</sub> was administered. This new substance makes possible the administration of large doses of the specific factor in concentrated form which may prove to be a distinct advantage in the treatment of patients who are resistant to treatment with liver extract or in patients with severe neurologic involvement where early in

tensive therapy is desirable. The isolation of an anti-pernicious anemia factor from the secretions of *Streptomyces griseus* is important in that this source may prove practical from the economical standpoint.

Vitamin B<sub>12</sub> is unique in that it is a cobalt-containing complex and has a characteristic purplish color. This places emphasis on the problem of the fundamental importance of cobalt as a trace substance in human nutrition.<sup>21</sup> Cobalt may be an element essential to the activity of vitamin B<sub>12</sub>; however, the cobaltous ion by itself does not appear to have any activity when tested by the microbiologic method.<sup>9</sup> Furthermore, West<sup>22</sup> treated two patients with pernicious anemia using cobalt acetate (single dose of 500  $\mu$ g) and cobalt chloride (single dose of 150  $\mu$ g) without hematologic response. Whether cobalt has any value in the treatment of other hematologic diseases is yet to be demonstrated. We have administered cobalt chloride to 5 cases of hypoplastic anemia and 3 cases with leukemia in doses of 10 to 25 mg daily (table 8). We have not noticed any significant hematologic changes which could be attributed to the administration of cobalt in these diseases and Burchenal<sup>23</sup> has had similar experiences in hypoplastic anemia, aplastic anemia and leukemia.

#### SUMMARY

1. A clinical remission in one case of pernicious anemia in relapse treated with 50  $\mu$ g of vitamin B<sub>12</sub> from liver is reported. The patient was followed for 114 days after two doses of 25  $\mu$ g were given on successive days and a peak of 3,900,000 erythrocytes occurred on the eighty-sixth day.

2. Preliminary observations are reported in 2 patients with pernicious anemia in relapse treated with a vitamin B<sub>12</sub> derived from *Streptomyces griseus*; the first patient who had no neurologic complaints received 32  $\mu$ g while the second was given two doses of 32  $\mu$ g each because of severe, subacute, combined degeneration. A good hematologic response and a satisfactory clinical remission occurred in both cases. There was definite improvement neurologically in the second case.

3. The administration of 32  $\mu$ g of vitamin B<sub>12</sub> derived from *Streptomyces griseus* to each of 5 cases of pernicious anemia in remission resulted in minor subjective and objective improvement.

#### CONCLUSIONS

1. Vitamin B<sub>12</sub> from liver and vitamin B<sub>12</sub> derived from *Streptomyces griseus* produce similar clinical results when dosages of the same magnitude are given to patients with pernicious anemia in relapse. Since the physical and chemical properties are also very similar this implies that these substances are closely related or identical.

2. The results obtained in this study are consistent with previous indications that vitamin B<sub>12</sub> is the single or at least the principal active anti-pernicious anemia factor in liver extract.

3. If the active factor can be prepared economically from cultures of *Streptomyces griseus*, this may prove a valuable source for the specific therapeutic agent in pernicious anemia and related macrocytic anemias.

4. The ingestion of cobalt chloride has little or no effect upon the blood levels of cases of hypoplastic anemia and leukemia.

TABLE 8—*Effect of Ingestion of Cobalt Chloride upon some Hematologic Dyscrasias*

Name	Sex age color	Diagnosis	Daily dosage of cobalt chloride	Inclusive dates of oral ingestion	HB and RBC levels before cobalt		Dates and levels of HB and RBC after cobalt chloride intake		
					HB	RBC		HB	RBC
			mg		%	million		%	million
C. U.	F 66 W	Myelo- fibrosis	25	2-11-48	28	1.2	4-28-48	29	1.7
				8-18-48			10-17-48	36	1.6
			10	11-17-48			12-19-48	36	1.9
				1-19-49			1-19-49	30	2.2
A. P.	F 49 W	Hypo- plastic anemia	25	2-11-48	36	1.8	3-3-48	35	1.8
				8-18-48			5-16-48	31	1.5
							9-29-48	35	1.3
S. E.	F 64 W	Hypo- plastic anemia	25	2-11-48	44	2.6	3-17-48	33	1.5
				6-2-48			7-14-48	32	1.6
							11-10-48	33	1.8
							1-19-49	32	2.1
F. M.	M 19 W	Hypo- plastic anemia	25	4-11-48	26	1.2	4-30-48	24	1.0
				10-10-48			10-22-48	38	2.1
							1-12-49	45	2.5
L. D.	F 20 W	Hypo- plastic anemia	10	1-2-48 2-21-48	60	2.5	2-21-48	Died of cerebral hemorrhage without change in he- matological levels	
B. B.	F 45 W	Myeloid leuke- mia	25	3-11-48 4-30-48	58	3.5	6-9-48	Died of leukemia without change in hematological levels	
W. B.	M 6 W	Lymph- oid leuke- mia	10	3-26-48 4-10-48	48	2.5	4-18-48	Died of leukemia without change in hematological levels	
M. M.	M 58 W	Lymph- oid leuke- mia	25	3-4-48 3-24-48	46	3.6	4-16-48	Died of leukemia without change in hematological levels	

## ADDENDUM

The purpose of the addendum is to discuss I the further progress of the eight cases reported above, II the presentation of three additional cases of pernicious anemia treated with vitamin B<sub>12</sub> (from *Streptomyces griseus*), III the administration of vitamin B<sub>12</sub> sublingually, and IV additional comments

## I Interval Report of Cases Reported (Above)

Patient	Blood Count				Further Treatment	Comment
	Date (1949)	Day after 1st dose of vitamin B <sub>12</sub>	Hb (%)	RBC		
Mrs. A. O. B.	1-26	114	68.5	3.2	Liver ext 15 $\mu$ weekly since 1-26	Previous peak not exceeded since liver extract was started
A. C. (Case 1)	3-30	82	75.2	3.75	None	Remission lasted less than 130 days Sublingually (see below)
	5-18	131	55.8	2.60	B <sub>12</sub> 200 $\mu$ g	
	5-20	133				
K. R. (Case 2)	2-8	27	45	2.2	B <sub>12</sub> 25 $\mu$ g	Further neurologic improvement (see below) Remission waning 5-17
	4-27	105	83	4.4	B <sub>12</sub> 50 $\mu$ g	
	5-17	125	61	3.18		
F. P. (Case 3)	2-23	53	83.1	3.85	Liver ext 15 $\mu$ weekly since 2-23	Treatment restarted because of paresthesias even though blood count was normal
M. S. (Case 4)	5-4	133	72.2	3.5	None	Satisfactory remission maintained
A. N. (Case 5)	3-9	70	77.9	4.0	Liver ext 15 $\mu$ weekly since 3-9	Treatment restarted because of weakness and paresthesias although blood count was normal
M. B. (Case 6)	5-11	140	73.2	3.52	None	Satisfactory remission maintained
V. S. (Case 7)	4-7	85	58	2.4	B <sub>12</sub> 50 $\mu$ g	Sublingual administration of vitamin B <sub>12</sub> (see below)
	4-14	92	68	3.1	B <sub>12</sub> 100 $\mu$ g	
	5-2	110	68	3.3		

Case II, Mrs. K. R. The results of treatment of Mrs. K. R. (Case II) deserve further mention. On Feb. 8, 1949, the blood levels had risen to 45 per cent hemoglobin and to 2,200,000 erythrocytes. At this time an additional 25  $\mu$ g of B<sub>12</sub> (from *Streptomyces griseus*) were given by injection because of the severity of the neurologic lesions. There was a continuation of the highly satisfactory course with good strength and appetite and further improvement of the neurologic manifestations. On April 27, 1949, the station gait and position sense were normal as were the deep tendon reflexes. Only faint Hoffman's and Babinski's signs were present. The vibration sense had reappeared at the upper end of the tibiae (although still subnormal) and was faintly perceptible at the ankles. The blood levels reached a peak of 83 per cent hemoglobin and 4,400,000 erythrocytes on April 27, 1949. However, three weeks later there were early signs of relapse with slight regression of the neurologic improvement and a drop in the blood levels.

to 61 per cent hemoglobin and 3 180 000 erythrocytes. The remission (produced by a total dose of 89  $\mu$ g of B<sub>12</sub>) lasted therefore over 100 days. A dose of 50  $\mu$ g of B<sub>12</sub> was given intramuscularly on May 17 1949.

## II Report of Three Additional Cases

**Case 8** M. McC. a 64 year old Irish woman was admitted to the Jefferson Hospital Feb. 15 1949 with pernicious anemia in relapse. In addition to the usual history of weakness fatigue dyspnea anorexia weight loss and sore tongue the patient had noted mild jaundice for three months. The patient had a smooth tongue cheilososis and a palpable liver. The only neurologic change was diminution of vibration sense at the left ankle. The blood levels on admission were 28.8 per cent (4.4 Gm) hemoglobin and 1 300 000 erythrocytes. There was a reticulocytosis of 10.5 per cent because of ingestion of liver pills just before admission. On Feb. 28 1949 when the reticulocyte level had dropped to 3.2 per cent 50  $\mu$ g of B<sub>12</sub> (from *Streptomyces griseus*) were given intramuscularly. The reticulocyte peak was 16.6 per cent on the 5th day. There was remarkable increase in strength and appetite and the jaundice disappeared. Normal vibration sense was restored. The maximum hemoglobin level (70.8 per cent) and erythrocyte count (3 510 000) occurred on April 13 1949 (44 days after injection). Two weeks later April 27 1949 the blood levels had dropped (hemoglobin—60 per cent erythrocytes—3 000 000). The patient was then given 50  $\mu$ g of vitamin B<sub>12</sub> sublingually (See below.)

**Case 9** V. P. a 36 year old Negress was admitted to the Jefferson Hospital on March 12 1949 with pernicious anemia in relapse. The main clinical features included gastrointestinal disturbances cardiovascular complaints sore tongue anorexia weakness headaches and weight loss. The blood count was hemoglobin 21.3 per cent (3.29 Gm/100 cc) erythrocytes 950 000 leukocytes 2 300 platelets 44 000 (per cu mm). The administration intramuscularly of 50  $\mu$ g of vitamin B<sub>12</sub> (from *Streptomyces griseus*) on March 15 1949 resulted in a reticulocyte peak of 39.2 per cent on the 6th day. The gastrointestinal complaints subsided within two days and the patient developed an excellent appetite. By April 20 1949 the atrophy of the tongue had completely disappeared as had also the cardiac signs and symptoms. Increase in strength paralleled the gradual rise of blood levels which reached 64.5 per cent hemoglobin and 3 800 000 erythrocytes on May 4 1949 the 45th postinjection day. The blood count was maintained at this level and the patient felt well when she was last seen on May 18 1949.

**Case 10** J. G. a 47 year old white man was admitted to the Jefferson Hospital March 2 1949 with a spastic unsteady gait and weakness of the lower extremities. A diagnosis of pernicious anemia in relapse (hemoglobin 35 per cent erythrocytes 1 400 000) had previously been made in March 1948 and a hematologic remission had been produced and maintained by regular injections of liver extract. The neurologic picture was complicated by an old spinal cord injury (incurred in 1938) for which he had been observed by the Neurology Service since 1946. This injury had resulted in an hypotonic bladder a residual partial Brown-Sequard syndrome on the left side at the level of T8 and a partial posterior-column syndrome with loss of vibration sense from the lower ribs down but intact position sense. The patient's spasticity had its onset with the anemia and had become progressively worse until the time of admission. Diagnosis was made of lateral-column disease secondary to pernicious anemia. On March 15 1949 50  $\mu$ g of vitamin B<sub>12</sub> (from *Streptomyces griseus*) were given intramuscularly and a dose of 25  $\mu$ g each week thereafter. There was an early improvement in steadiness of gait and a gradual decrease of spasticity. The hyperactivity of the reflexes lessened as did the intensity of the pathologic reflexes in all extremities. At the end of eight weeks the functional performance was reasonably good. There was no improvement of the changes resulting from the old injury.

## III Sublingual Administration of Vitamin B<sub>12</sub>

Pernicious anemia seems to be a disease of impaired absorption (through the gastrointestinal mucosa) of fats carbohydrates proteins<sup>11</sup> and certain vitamins, particularly vitamin B<sub>12</sub>. Hall et al.<sup>12</sup> have shown that dosages (25  $\mu$ g) of B<sub>12</sub> which are effective when given intramuscularly, are ineffective when given orally, unless normal gastric juice is added. This suggests that the intrinsic factor acts merely as a carrier or absorber of B<sub>12</sub> through the gastrointestinal mucosa.

However, West<sup>16</sup> stated that large doses (600  $\mu\text{g}$ ) of  $\text{B}_{12}$  given orally will produce a maximum reticulocyte response. This would imply either that patients with pernicious anemia make small but insufficient quantities of intrinsic factor (permitting only partial absorption of available  $\text{B}_{12}$ ), or that  $\text{B}_{12}$  in relatively high concentration is absorbed by mass action (despite the lack of absorber or intrinsic factor). Realizing that saliva does not contain intrinsic factor, we were interested in determining if vitamin  $\text{B}_{12}$  concentrated on a small area of oral mucosa would be appreciably absorbed. Hence three patients were given  $\text{B}_{12}$  sublingually.

*Case 1.* A. C. On May 18, 1949 the blood levels had dropped to 55.8 per cent hemoglobin and 2,600,000 erythrocytes; the reticulocyte count was 0.7 per cent. On May 20, 1949 the patient received sublingually 100  $\mu\text{g}$  of vitamin  $\text{B}_{12}$ . The reticulocyte level rose to 4.2 per cent on May 23, 1949.

*Case 7.* V. S. was given 50  $\mu\text{g}$  of vitamin  $\text{B}_{12}$  sublingually on April 7, 1949 at which time the hemoglobin was 58 per cent, the erythrocyte count 2,400,000 and the patient complained of weakness and parasthesias of the hands and feet. On the 5th day after the administration the reticulocyte level had increased to 5.2 per cent. The patient felt much improved. On April 14, 1949 another 100  $\mu\text{g}$  were given sublingually. On May 2, 1949 the patient continued to feel well and the blood count was 68 per cent hemoglobin with 3,300,000 erythrocytes per cu. mm.

*Case 8.* M. McC. was given 50  $\mu\text{g}$  of vitamin  $\text{B}_{12}$  sublingually on April 27, 1949 when it appeared that her count had started to drop. The hemoglobin at this time was 60.6 per cent (9.33 Gm) and the erythrocyte count was 3,000,000. The patient noticed stiffness of the hands and parasthesias and there was diminished vibration sense again at the left ankle. The tongue showed early atrophic changes. There was no reticulocytosis after the sublingual administration. However, the patient felt much better. One week later the stiffness of the hands had subsided but the decreased vibration sense persisted. In two weeks, May 11, 1949 the hemoglobin had risen to 69.2 per cent (11.67 Gm) and the erythrocyte count to 3,800,000. The vibration sense in the ankle became normal although occasional parasthesias were still present. The patient felt well except for a sore tongue which had developed during the second week. The end of the tongue was beefy red and the central and posterior portions atrophic. At this time, May 11, 1949 50  $\mu\text{g}$  of vitamin  $\text{B}_{12}$  were given intramuscularly. Again there was noted no reticulocytosis on the 5th day. The glossitis disappeared one week after injection.

#### IV. Comments

It is interesting to note that Addison exactly 100 years ago first accurately described pernicious anemia.<sup>17</sup> The isolation of the antipernicious anemia factor (the most potent therapeutic agent known to man at present) is a most appropriate centennial event.

Allowing for variations in the degree and type of relapse and in the individuals, responsiveness it can be estimated that the injection of 50 to 100  $\mu\text{g}$  of vitamin  $\text{B}_{12}$  is capable of producing a remission of pernicious anemia lasting 50 to 100 days, about 50  $\mu\text{g}$  given to a patient in remission will maintain the remission 70 to 120 days. The general complaints (weakness, fatigue and glossitis) and neurologic complaints (parasthesias and stiffness) appeared more difficult to control than the blood levels.

Treatment of pernicious anemia with  $\text{B}_{12}$  (or liver extract) occasionally results in iron deficiency during the development of remission. Thus one of our patients (K. R.) developed hypochromia of erythrocytes which was overcome with the use



of intravenously administered iron (saccharated iron oxide\*) Other deficiencies during remission in pernicious anemia are indicated by the work of Brown<sup>18</sup> who found that glossitis (and other lesions) developing during liver extract therapy required specific treatment, with pantothenic acid, nicotinic acid, riboflavin or folic acid Glossitis which developed in one of our patients, M McC, (while remission was complete in all other respects) responded well to an additional dose of B<sub>12</sub> As yet there have been no reported failures of glossitis to respond to adequate treatment with B<sub>12</sub>

The results of the sublingual therapy were inconclusive because of the difficulty in evaluating response in patients with partial remission Slight reticulocytosis occurred in two of the three trials and a definite clinical improvement seemed evident in each case The degree of response and the sites (gastric or sublingual) of absorption were uncertain

#### REFERENCES

- <sup>1</sup> BERK L DENNY BROWN D FINLAND M D, AND CASTLE, W B Effectiveness of vitamin B<sub>12</sub> in combined system diseases *New England J Med* 239 328, 1948
- <sup>2</sup> BURCHENAL J H Erythropoietic effect of cobalt in patients with or without anemia *New England J Med* 240 754, 1949
- <sup>3</sup> DAMESHEK W Editorial — And now B<sub>12</sub> *Blood* 4 76 1949
- <sup>4</sup> HALL, B E AND CAMPBELL, D C Vitamin B<sub>12</sub> therapy in pernicious anemia II Effect on the general clinical and neurologic manifestations Preliminary report *Proc Staff Meet Mayo Clinic* 23 591 1948
- <sup>5</sup> — AND — Vitamin B<sub>12</sub> therapy in pernicious anemia I Effect on hematopoietic system Preliminary report *Proc Staff Meet Mayo Clinic* 23 584 1948
- <sup>6</sup> LE GOFF J M Cobalt therapy *Gaz des hop de Paris* 120 381 1947
- <sup>7</sup> Miscellaneous Recommended terms and definitions for cells of the leukocytic erythroblastic and thrombocytic series *J A M A* 139 175 1949
- <sup>8</sup> RICKES E L BRINK N G KOVACSZY F R WOOD J R AND FOLKERS L Comparative data on vitamin B<sub>12</sub> from liver and from a new source *Streptomyces griseus* *Science* 108 634 1948 (D-c 3).
- <sup>9</sup> — — — — — AND — Vitamin B<sub>12</sub> a cobalt complex *Science* 108 134 1948
- <sup>10</sup> SHORR, M S Activity of vitamin B<sub>12</sub> for the growth of *Lactobacillus lactis* *Science* 108 397 1948
- <sup>11</sup> SPIES J D STONE R E AND ARAMBURU J Observations on the antianemic properties of vitamin B<sub>12</sub> *South M J* 41 522, 1948
- <sup>12</sup> WEST R Activity of vitamin B<sub>12</sub> in Addisonian pernicious anemia. *Science* 107 398 1948
- <sup>13</sup> WINTROBE M M *Clinical Hematology* ed L Lea & Febiger, Philadelphia 1947 P 331
- <sup>14</sup> ERF L A AND RHODES C P The glycine tolerance test in sprue and pernicious anemia *J Clin Investigation* 19 409 1940
- <sup>15</sup> HALL B E MOROAN E H AND CAMPBELL, D C Oral administration of vitamin B<sub>12</sub> in pernicious anemia *Proc Staff Meet Mayo Clin* 24 99 1949
- <sup>16</sup> West R Paper presented at Atlantic City on May 3 1949
- <sup>17</sup> — — — — — AND REISNER E. H JR Treatment of pernicious anemia with crystalline vitamin B<sub>12</sub> *Am J Med* 6 643, 1949
- <sup>18</sup> ADDISON W Anemia-disease of the suprarenal capsules *London Med Gazette* 43 517 1849
- <sup>19</sup> BROWN, A L Glossitis in Addisonian pernicious anemia *Brit M J*, 1 704 1949

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\* This preparation supplied to us by Dr Edwin McLean of Smith Kline and French Laboratories Philadelphia

# THE EFFECT OF VARIOUS ANTICOAGULANTS ON THE SPECIFIC GRAVITY OF BLOOD AND OF PLASMA, AND ON THE HEMATOCRIT

By PAUL L. McLAIN, M.D., AND C. H. WILLIAM RUIE, M.D.,  
WITH GEORGE J. PASTORIUS, M.D.

THE INTRODUCTION of rapid, simple methods for measuring the specific gravity of body fluids<sup>1-3</sup> has led to a growing appreciation of the usefulness of such measurements, particularly as applied to blood and plasma<sup>2-10</sup>. Although it is not difficult to measure the specific gravity of blood prior to coagulation, the comparable treatment of plasma requires special facilities for rapid sedimentation of the corpuscles. In general, the measurements are carried out much more conveniently on samples which have been defibrinated or in which coagulation has been prevented by the use of various chemicals. The influence of certain anticoagulant procedures on the specific gravity of blood and plasma has been reported,<sup>2, 11, 12</sup> but greater emphasis has been placed upon the effects on red cell volume<sup>12-24</sup>. In the observations here reported, the specific gravity of defibrinated, heparinized, oxalated, and citrated blood, as well as that of the serum or plasma from blood so treated, was compared with the specific gravity of an aliquot of the same sample, untreated and prior to coagulation. Similar comparisons were made for relative red cell volumes by hematocrit.

## MATERIAL, PROCEDURE AND METHODS

All observations were made on arterial blood freshly drawn from rabbits. The animals were anesthetized lightly with ether and a large bore hypodermic needle was tied into one of the carotid arteries exposed in the neck. Approximately 20 ml. of blood were withdrawn into a syringe and the blood was then quickly transferred in measured amounts to small, appropriately prepared test tubes. The drawing and transfer of the blood required only a few seconds; hence uniformity of the sample was assured.

Plasma from untreated blood was obtained by centrifugalizing freshly drawn blood for thirty seconds in an angle centrifuge at about 12,000 revolutions per minute (mean radius 6.7 cm.). Aliquots for defibrination were stirred with fine nickel silver wires for the required length of time, usually about five minutes. Samples for chemical anticoagulant treatment were mixed promptly with the proper amounts of reagent previously placed in the receptacles. The anticoagulants used with their respective amounts for each ml. of blood, were as follows: heparin dry, 0.1 mg. (10 units); heparin, solution 0.01 ml. (equivalent to 0.1 mg. of dry heparin); sodium citrate powdered 5.0 mg.; potassium oxalate, powdered 2.0 mg.; potassium oxalate 1.6 per cent solution 0.125 ml. (equivalent to 2.0 mg. of dry potassium oxalate); ammonium potassium oxalate mixture<sup>25</sup> 3.2 by weight 1.0 mg.

The specific gravity (25/25 C.) of the blood and of the plasma or serum for each of the above conditions was measured by a modification of the falling drop method of Barbour and Hamilton.<sup>1</sup> Relative volumes of red cells and plasma or serum were measured centrifugally by an hematocrit of the Daland<sup>26</sup> type operated at 12,000 r.p.m. and 4.7 cm. effective radius (R.C.F. approximately 7,500 x G.) until constant sediment volumes were obtained.

From the Department of Physiology and Pharmacology, School of Medicine, University of Pittsburgh, Pittsburgh, Penna.

Presented in part before the Twenty ninth Annual Meeting of the Federation of American Societies for Experimental Biology, Boston, Mass., March 31-April 4, 1942.

## RESULTS AND DISCUSSION

Analysis of the data by the usual statistical methods showed that, for the distributions obtained, demonstration of heterogeneity among the various series of observations required differences far in excess of the established experimental errors for the methods employed. For this reason, the occurrence of certain minimum differences between treated and untreated aliquots of individual samples was considered a more reliable basis for analysis than the conventional difference between means, especially for limited series. The tables, therefore, show the frequency and direction (a) of all changes which occurred in the individual comparisons, and (b) of all changes in excess of somewhat arbitrary limits, chosen to represent changes measurable with certainty by the methods employed, yet of limited or doubtful significance when interpreted clinically, especially in view of the magnitude of normal diurnal variations. In addition, mean and algebraic mean changes are tabulated.

*Effect of Anticoagulant Procedures on Specific Gravity*

**Blood.** The first section of table 1 summarizes the changes in specific gravity of whole blood associated with the various anticoagulant procedures. All but two of these produced mean changes in excess of 0.0004, the limit chosen as representing a significant difference from the control specific gravity. The greatest changes, as well as the most frequent and most consistent in direction, occurred under the influence of sodium citrate and dry potassium oxalate. Both of these chemicals, in the amounts employed, increased the specific gravity of blood at every trial. Further, all but one such increase exceeded 0.0004. Dry heparin increased the specific gravity in a significant majority of cases, though an occasional decrease occurred. Increases greater than 0.0004 were noted in about half the cases, and the algebraic mean change was positive in sign and just below the limit of significance. Heparin solution, on the other hand, decreased the specific gravity in all but one instance, and more than two-thirds of these decreases were greater than 0.0004. Correction of the results for the volume and specific gravity of the heparin solution (not shown in the table) materially reduced the number of significant changes, and reduced the mean change by approximately one-half. However, such correction is awkward and impractical for routine work. Potassium oxalate in 1.6 per cent solution is generally considered to be isosmotic with blood,<sup>21</sup> and should therefore produce little or no change in corpuscle size. Further, it should be possible to correct observed specific gravities for the volume and specific gravity of the oxalate solution added to blood as an anticoagulant. In this series, values so corrected were lower than the controls by more than 0.0004 in half the trials and within 0.0004 of the control specific gravity in the remainder. The correction was therefore only 50 per cent effective. The Heller-Paul oxalate mixture caused increases and decreases in specific gravity with almost equal frequency. However, relatively few of these changes exceeded 0.0004, and the algebraic mean change was +0.0001, which indicates the best distribution of changes in the entire series of observations. Defibrination caused the smallest mean change in specific gravity of blood and gave the greatest frequency of values identical with the control. With regard to the fre-

quency of significant changes, defibrination was very nearly equivalent to oxalate mixture

Of the anticoagulant procedures tested, then, defibrination and addition of Heller-Paul oxalate mixture caused the smallest and least frequent changes in the specific gravity of whole blood. Since both positive and negative changes occurred,

TABLE 1—*Changes in Specific Gravity of Blood Plasma and Corpuscles Caused by Various Anticoagulant Procedures*

Procedure	Number	Frequency of Changes in Specific Gravity			Frequency of Changes Greater than 0.0004			Changes $\times 1000$	
		In creased	De creased	Un changed	In creased	De creased	Un changed	Mean	Alg. Mean
		%	%	%	%	%	%		
<i>Blood</i>									
Defibrination	15	40.0	26.7	33.3	20.0	6.7	73.3	0.30	+0.19
Heparin, dry	9	66.7	22.2	11.1	55.6	22.2	22.2	0.69	+0.38
Heparin solution	11	0.0	90.9	9.1	0.0	63.6	36.4	0.73	-0.73
Na Citrate	18	100.0	0.0	0.0	100.0	0.0	0.0	2.28	+2.28
K Oxalate, dry	20	100.0	0.0	0.0	95.0	0.0	5.0	1.07	+1.07
K Oxalate, 1.6% sol.†	14	21.4	71.5	7.1	0.0	50.0	50.0	0.73	-0.61
Oxalate Mixture	9	55.6	44.4	0.0	22.2	0.0	77.8	0.37	+0.10
<i>Plasma (or Serum)</i>									
Defibrination	9	11.1	77.8	11.1	0.0	22.2	77.8	0.29	-0.20
Heparin, dry	9	88.9	0.0	11.1	77.8	0.0	22.2	0.66	+0.66
Na Citrate	10	100.0	0.0	0.0	100.0	0.0	0.0	3.28	+3.28
K Oxalate, dry	10	100.0	0.0	0.0	100.0	0.0	0.0	1.26	+1.26
K Oxalate, 1.6% sol.†	10	60.0	30.0	10.0	50.0	10.0	40.0	0.61	+0.22
Oxalate Mixture	10	100.0	0.0	0.0	100.0	0.0	0.0	1.07	+1.07
<i>Corpuscles†</i>									
Defibrination	8	50.0	50.0	0.0	0.0	0.0	100.0	1.16	+0.28
Heparin, dry	8	62.5	25.0	12.5	12.5	12.5	75.0	1.99	+0.66
Na Citrate	9	100.0	0.0	0.0	100.0	0.0	0.0	9.28	+9.28
K Oxalate, dry	9	100.0	0.0	0.0	100.0	0.0	0.0	6.47	+6.47
K Oxalate, 1.6% sol.†	9	22.2	77.8	0.0	0.0	11.1	88.9	1.27	-0.98
Oxalate Mixture	9	11.1	88.9	0.0	0.0	22.2	77.8	2.11	-1.93

\* For corpuscles read 0.0029 in place of 0.0004 (see text)

† Observed specific gravities were corrected for volume and specific gravity of added solution.

‡ Specific gravities of corpuscles were calculated

and since the mean change was not significant from a practical standpoint, correction of observed specific gravities for the effects of these two procedures is not recommended

*Plasma* The second section of table 1 shows the influence of the various anticoagulant methods on the specific gravity of plasma. As in the case of whole blood, sodium citrate and dry potassium oxalate increased the specific gravity in all trials and to a highly significant degree. Oxalate mixture, which had insignificant effects on the specific gravity of whole blood, increased that of plasma markedly in all

cases Dry heparin increased the specific gravity of plasma in a clear majority of instances, the effect being slightly more pronounced on plasma than on whole blood. The effect of oxalate solution, after appropriate correction, was to increase the specific gravity in excess of 0.0004 in half the cases, the mean change being 0.00061. While serum from defibrinated blood had a lower specific gravity than that of the corresponding plasma in nearly 80 per cent of trials, the differences exceeded 0.0004 in only 22 per cent, the mean change being 0.00029. There were no direct comparisons between untreated plasma and that resulting from the use of heparin solution as an anticoagulant. However, the mean difference between such plasma and serum from defibrinated blood was insignificant. Since dry heparin increased the specific gravity of plasma while defibrination tended to reduce it, the heparin-solution as here employed seemed satisfactory for preservation of the specific gravity of plasma, but not of blood.

TABLE 2.—*Changes in Hematocrit Caused by Various Anticoagulant Procedures*

Procedure	Number	Frequency of Changes in Hematocrit			Frequency of Changes Greater than 1.0 Vol %			Changes in Hematocrit	
		In creased	De creased	Un changed	In creased	De creased	Un changed	Mean	Alg Mean
		%	%	%	%	%	%	Vol %	Vol %
Defibrination	13	61.5	15.4	23.1	23.1	0.0	76.9	0.73	+0.58
Heparin dry	10	10.0	70.0	20.0	0.0	0.0	100.0	0.50	-0.49
Heparin solution	4	0.0	75.0	25.0	0.0	0.0	100.0	0.63	-0.63
Na Citrate	14	0.0	100.0	0.0	0.0	100.0	0.0	3.75	-3.75
K Oxalate dry	14	0.0	100.0	0.0	0.0	92.9	7.1	2.36	-2.36
K Oxalate, 1:6% sol *	14	35.7	35.7	28.6	0.0	7.1	92.9	0.57	-0.07
Oxalate Mixture	10	40.0	20.0	40.0	0.0	0.0	100.0	0.40	+0.20

\* Observed values corrected for volume of added solution

**Corpuscles** The specific gravity of red cells was calculated from the observed specific gravities of whole blood and plasma, and the relative volumes of cells and plasma obtained by hematocrit. The results were therefore influenced by the combined errors of the basic measurements. Computations indicated that a change in corpuscle specific gravity of 0.003 or greater should be detectable with certainty even though the contributory errors might be additive. All changes in corpuscle specific gravity greater than 0.0029 were, hence, considered significant.

The third section of table 1 shows that sodium citrate and dry potassium oxalate increased the specific gravity of corpuscles in all observations. The remaining anticoagulant procedures produced few significant effects.

#### *Effect of Anticoagulant Procedures on the Relative Volumes of Corpuscles and Plasma*

The hematocrit employed in this study was capable of revealing with certainty changes in excess of 1.0 volume per cent. Among the anticoagulant procedures, sodium citrate and dry potassium oxalate consistently produced marked shrinkage of the red cell volume, as shown in table 2. Among the other methods, only occasional changes in excess of 1.0 volume per cent occurred. No such changes were

noted with oxalate mixture, dry heparin, or heparin solution, and only one with 1.6 per cent potassium oxalate solution after correction for volume of added fluid. Defibrination tended to increase the hematocrit results, though the change was less than 1.0 volume per cent in over three-fourths of the trials. The best methods for preservation of cell-plasma volume relationships were oxalate mixture and isosmotic potassium oxalate solution.

#### SUMMARY

The effects of several commonly employed anticoagulant procedures on the specific gravity of blood and of plasma, and on the relative red cell volume, were studied in freshly drawn samples of arterial blood from rabbits. Measurements on treated blood, or its fluid component, were compared with corresponding results on portions of the same samples without treatment and prior to coagulation.

Satisfactory preservation of the specific gravity of whole blood was obtained by defibrination or by the use of ammonium-potassium oxalate mixture.

Satisfactory preservation of the specific gravity of plasma was obtained by defibrination or by the use of heparin solution.

The relative volume of red cells was essentially unaltered by the use of dry heparin, oxalate mixture, 1.6 per cent solution of potassium oxalate, or by defibrination.

Dry potassium oxalate and sodium citrate caused marked changes, increasing the specific gravity of blood and of plasma, and shrinking the red cells.

Dry heparin caused significant increases in the specific gravity of blood and of plasma.

Ammonium-potassium oxalate mixture increased the specific gravity of plasma markedly and consistently.

#### REFERENCES

- <sup>1</sup> BARBOUR H G AND HAMILTON, W F. Blood specific gravity. Its significance and a new method for its determination. *Am J Physiol* 69 654 1924.
- <sup>2</sup> PHILLIPS R A, VAN SLYKE, D D, DOLE V P, EMERSON, K JR, HAMILTON P B AND ARCHIBALD R M. The copper sulfate method for measuring the specific gravities of whole blood and plasma. *Bull U S Army Med Dept* 71 66 1943.
- <sup>3</sup> —, —, —, —, AND —. Copper sulfate method for measuring specific gravities of whole blood and plasma. Monograph from the U S Navy Research Unit at the Hospital of the Rockefeller Institute for Medical Research (undated).
- <sup>4</sup> MOORE N S AND VAN SLYKE D D. The relationships between plasma specific gravity, plasma protein content and edema in nephritis. *J Clin Investigation* 8 337 1930.
- <sup>5</sup> WEECH A A, REEVES E B AND GOETTSCH E. The relationship between specific gravity and protein content in plasma, serum and transudate from dogs. *J Biol Chem* 113 167 1936.
- <sup>6</sup> SCUDDER J. Shock. Blood Studies as a Guide to Therapy. Philadelphia J B Lippincott Co 1940.
- <sup>7</sup> ASHWORTH C T AND TIOERTT W D. A simple rapid method for determining relative blood volume changes by specific gravity studies. *J Lab & Clin Med* 26 1545, 1941.
- <sup>8</sup> — AND ADAMS G. Blood specific gravity studies. Relationship of specific gravity of whole blood to specific gravity of plasma, red blood cell count, hematocrit and hemoglobin as indicators of hemoconcentration. *J Lab & Clin Med* 26 1934 1941.
- <sup>9</sup> GRAY P A AND ELLIOTT A H. Specific gravity of whole blood and serum. *Am J M Sc* 205 356, 1943.

- <sup>10</sup> PHILLIPS R A YEOMANS A DOLE V P FARR L E AND VAN SLYKE D D WITH HOGAN D  
Estimation of blood volume from change in blood specific gravity following a plasma infusion J  
Clin Investigation 25 262 1946
- <sup>11</sup> McLAIN P L PASTORIUS G J AND RUKE C H W Effect of anticoagulants on the specific gravity  
of blood and plasma Fed Proc 1 52 1942
- <sup>12</sup> COPLEY A L AND WHITNEY M E Density findings with heparin in blood plasma and serum J  
Lab & Clin Med 28 1501 1943
- <sup>13</sup> HOOVER C W SMITH H P BELT, A E, AND WHIFFLE G H Blood volume studies I Experi-  
mental control of a dye blood volume method Am J Physiol 55 205 1920
- <sup>14</sup> VAN ALLEN C M. An hematocrit method J Lab & Clin Med 20 1027, 1925
- <sup>15</sup> HADEN R L The technic of determination of the relative mass the individual cell volume and the  
volume index of the erythrocytes of man J Lab & Clin Med 25 736 1930
- <sup>16</sup> MAGATH T B Concerning anticoagulants Am J Clin Path 5 548 1935
- <sup>17</sup> HIGOUNET H Influence des anticoagulants sur la repartition des ions Cl entre le plasma et les hema-  
ties Bull Soc Chim Biol 29 53 1937
- <sup>18</sup> ETTORI J AND GRANOUD R Influence of anticoagulants on plasma-cell proportion Compt rend  
Soc de biol 128 181 1938
- <sup>19</sup> MARENZI A D AND LIDA E Volume of red blood cell Influence of anticoagulant concentration  
Rev Soc argent Biol 25 217 1939
- <sup>20</sup> NAPIER L E The choice of an anticoagulant Indian J Med Research 27 605 1939
- <sup>21</sup> LEICHENRING J M DONELSON E G WALL L M AND OHLSON M A Evaluation of oxalate solu-  
tions for determination of packed cell volume in human blood J Lab & Clin Med 25 35, 1939
- <sup>22</sup> PETERS J P AND VAN SLYKE D D Quantitative Clinical Chemistry Vol II Methods Baltimore  
Williams and Wilkins Co 1932 P 61
- <sup>23</sup> PONDER E The Mammalian Red Cell and the Properties of Haemolytic Systems Berlin Gebrüder  
Borntraeger 1934
- <sup>24</sup> WINTROBE M M Clinical Hematology Philadelphia Lea & Febiger 1943 P 179
- <sup>25</sup> HELLER V G AND PAUL H Changes in cell volume produced by varying concentrations of differ-  
ent anticoagulants J Lab & Clin Med 29 777 1934
- <sup>26</sup> DALAND J Ueber das Volumen der roten und weissen Blutkörperchen im Blute des gesunden und  
kranken Menschen Fortschr d Med Berlin 9 867 1891

# THE KELL-CELLANO (K-k) GENETIC SYSTEM OF HUMAN BLOOD FACTORS

By PHILIP LEVINE, M D , MILTON WIGOD, M A , ABBY M BACKER, M D ,  
AND RUTH PONDER, B A

A NEW agglutinable factor of human blood was recently discovered with the aid of an antibody, an agglutinin, which is remarkable for its high incidence of positive reactions, 99.8 per cent, in white individuals (U S A ) This antibody was found in a mother, Mrs Cellano, whose infant had a mild form of hemolytic disease Accordingly, the antibody will be referred to as anti-Cellano and its corresponding agglutininogen as Cellano

A study of the family of an individual from among the 0.2 per cent Cellano negative group revealed the hereditary nature of the Cellano factor \* Of the eight children, three were Cellano negative Since both parents were Cellano positive, they were presumed to be heterozygous Assuming two allelic genes, one determining Cellano positive and the other Cellano negative, the following values for the three genotypes were derived †

Homozygous Cellano Positive	91.2%
Heterozygous Cellano Positive	8.6%
Homozygous Cellano Negative	0.2%

Because of the possible analogy to the M-N and the three Rh-Hr systems, the authors considered the existence of another genetically related blood property present in the Cellano negative and Cellano heterozygous groups Thus, this theoretic blood factor should have incidences of about 8.8 per cent Two types of antibodies giving very similar incidences (anti-Lutheran 8 per cent and anti-Kell 7 per cent) had been observed by Coombs, Mourant, Race and their co-workers <sup>2,3,4</sup> An analysis of the results with anti-Cellano and two specimens of anti-Kell sera on the above-mentioned family reveals the significant conclusion that the Cellano and Kell antigens are genetic alleles (table 1)

For the sake of uniformity, the letters K and k, already employed by the British workers for the genes determining Kell positive and Kell negative reactions, respectively, will be retained The results given in table 1 indicate that the gene k can now be considered as indicating the presence of the Cellano factor

As shown in table 1, both parents are heterozygous (Kk) and each is capable of transmitting the genes K and k to their offspring Such matings, which occur very rarely, i e , 8.6 per cent  $\times$  8.6 per cent or one in 135, are most useful for analysis of

From the Rh Blood Testing Laboratory Ortho Research Foundation Raritan New Jersey and the Nassau Hospital Laboratory Mineola N Y

\* See table 1 and the paper by Levine et al <sup>1</sup>

† The incidence of the gene for Cellano negative =  $\sqrt{0.002} = 0.045$  or 4.5% while the incidence of the gene for Cellano positive =  $1 - 0.045 = 0.955$  or 95.5% The three values given above are derived from the equation  $(.955 + .045)^2$



factors which have either very low or very high incidences. Thus, Cellano negatives of genotype KK (homozygous Kell) which should occur in 25 per cent of the offspring, were found in three of the eight children in contrast to 0.2 per cent in a random population. Similarly, Kell positive (KK and Kk) which should occur in 75 per cent of the offspring of heterozygous parents, were found in six of the eight children in contrast to 88 per cent in a random population.

Further evidence for the allelic nature of the Kell and Cellano factors was obtained in a study of the five siblings of another Cellano negative individual. Two of the five are Cellano negative and four are Kell positive. These findings are presented in table 2.

TABLE 1

	Anti Kell Anti Lazarus	Anti Cellano	Genotype
Father Mr J. Kul Sr	+	+	Kk
Mother Mrs M. Kul	+	+	Kk
Children 1 John	+	+	Kk
2 Julian	+	o	KK
3 Mrs A. G.	o	+	kk
4 Mrs I. S.	+	+	Kk
5 Mrs A. V.	+	o	KK
6 Frank	+	o	KK
7 Andrew	+	+	Kk
8 Josephine	o	+	kk
Controls Mrs Cellano	+	o	KK
Mr Lazarus	+	+	Kk
Mrs Lazarus	o	+	kk
Jeffrey Lazarus	o	+	kk

As in the Kul family, the parents of the five siblings must be heterozygous for both factors and this was confirmed when their bloods were subsequently tested.

In the first publication of the Kell antigen, its frequency was given as 7 per cent, while the serum studied by Wiener and Sonn reacted on 13 per cent. \* Sanger, Race and their co-workers now report a larger series with an incidence of 10.17 per cent positive reactions. The calculated incidences of three genotypes based on the latter value and on the value 99.8 per cent for the Cellano factor are compared in table 3.

The close agreement of these values further supports the view that the genes for the Kell and Cellano factors are allelomorphous to each other. As in the case of the M-N and the three Rh-Hr systems (Dd, Cc, and Ee), the three genotypes resulting from the interaction of these two genes correspond to the three phenotypes identified by parallel tests with the Kell and Cellano antibodies. A type of blood which fails to react with both sera has not been observed.

Sera containing anti-Cellano antibodies are necessarily very rare, while the anti-Kell type of antibody has been observed at least six times. A list of these follows.

\* The identification of this antibody as anti Kell was made by Dr. Race.

1 Kell	Coombs Mourant and Race <sup>1</sup>
2 Si	Wiener and Sonn <sup>2</sup>
3 Drizen	Sanger and Abelson <sup>3</sup>
4 And	Dunsford <sup>4</sup>
5 Lazarus	Levine Rauch and Block <sup>5</sup>
6 P L	Vogel and Rosenfeld <sup>10</sup>

TABLE 2

	Anti K (Anti Lazarus)	Anti k (Anti Cellano)	Genotype
Father Mr H Mc	+	+	Kk
Mother Mrs L. Mc	+	+	Kk
Children 1 Mrs B M	+	o	KK
2 J G Mc	+	+	Kk
3 E. V Mc	o	+	kk
4 Mrs A R	+	+	Kk
5 Mrs V S	+	o	KK

TABLE 3

	Anti Kell (Anti K)	Genotype	Anti Cellano (Anti k)
Kell positive	$\left\{ \begin{array}{l} 89 \ 83 \\ 9 \ 90 \\ 0 \ 27 \end{array} \right.$	$\left\{ \begin{array}{l} Kk \\ Kk \\ KK \end{array} \right.$	$\left. \begin{array}{l} 91 \ 2 \\ 8 \ 6 \\ 0 \ 2 \end{array} \right\} \text{Cellano positive}$

The greater incidence of anti-Kell type of sera is not surprising since incompatible matings for the Kell factor occur in  $8 \ 8 \times 91 \ 2$ , or  $1 \ 12 \ 5$  in contrast to a value of  $99 \ 8 \times 0 \ 2$  or  $1 \ 500$  for the Cellano factor. Thus, the opportunity for the production of anti-Kell sera is 40 times greater than for anti-Cellano.

Anti-Kell type of antibody may be missed in routine tests for isoimmunization, unless the mother's serum is tested against her husband's cells, suspended in bovine albumin, and with the Coombs technic. These procedures are essential for all instances in which isoimmunization may be brought about by a blood factor characterized by a low incidence in the general population. Anti-Kell may be differentiated from anti-Lutheran since the latter antibody does not give a positive Coombs test and, in contrast to anti-Kell, its reactions are stronger at lower temperatures than at 37°C.

Antibody of the anti-Cellano type may be expected if the serum gives an unusually high incidence of positive reactions. It is important, however, to exclude the anti-e (anti-hr<sup>+</sup>) antibody which gives about 97 per cent positive reactions, or the coexistence of more than one antibody. The latter possibility may be tested by suitable absorption experiments with carefully selected bloods of known antigenic structure.

Extensive racial and genetic studies will be carried out when larger supplies of these two antibodies become available. Preliminary experiments have shown that the Kell and Cellano factors are not antigenic in rabbits.

Although preliminary data indicate that Kell and Cellano factors are not related to other blood properties, more comprehensive studies are required to exclude the possibility of linkage.

#### ACKNOWLEDGMENT

The authors are indebted to R. R. Race for a sample of anti Kell serum. With the aid of this specimen it was possible to identify another antibody (anti Lazarus) studied in our laboratory since 1946 as of the Kell variety. This patient had two stillbirths due to hemolytic disease of the fetus and one surviving child who is Kell or Lazarus negative, the husband being heterozygous (cf. controls in table 1). The authors are also indebted to Mr. Benson Rosenberg, Elizabeth, N. J. for the blood specimens of the Kul family and to Dr. W. E. Hoffman, Charleston, W. Va. for the Mc family.

The tests with anti Kell were made with the aid of Coombs anti human serum. Identical results with anti Lazarus serum were obtained using both the Coombs technique and albumin suspended cells. The test with anti-Cellano were made with saline suspended cells.

#### REFERENCES

- <sup>1</sup> LEVINE P, BACKER M, WOOD M AND PONDER R. *Science* in press.
- <sup>2</sup> CALLENDAR S T, RACE R R AND PAYKOC Z V. *Brit M J* 2: 83, 1945.
- <sup>3</sup> COOMBS R R, MOURANT A E and RACE R R. *Lancet* 1: 264, 1946 (Case 14).
- <sup>4</sup> RACE R R. *Brit Med Bulletin* 4: 188, 1946.
- <sup>5</sup> SANGER R, BERTINSHAW D, LAWLER S D AND RACE R R. *Rev d Hemat* in press.
- <sup>6</sup> LEVINE P, RAUCH E AND BLOCK M. In preparation.
- <sup>7</sup> WIENER A S AND SONN-GORDON E B. *Rev d Hemat* 2: 1, 1947.
- <sup>8</sup> SANGER R AND ABELSON N. Cited by Race in personal communication.
- <sup>9</sup> DUNSFORD I. *Nature* in press.
- <sup>10</sup> VOGEL, P AND ROSENFELD R. In preparation.

## EDITORIAL

### AUTO-IMMUNIZATION

BETWEEN 1907 and 1914, the [French workers Chauffard<sup>1</sup> Widal and their collaborators discriminated sharply between hereditary and acquired types of hemolytic anemia. The rivalry between these two groups was keen, but from it sprang many new concepts and diagnostic tests. Widal, Abrami, and Brule found auto-agglutinins in each of their cases of acquired hemolytic anemia and pointed to the diagnostic value of this finding. The significance of their observations was lost for many years, and for a long period (approximately between 1920 to 1940) many observers stated that no real distinction could be made between hereditary and acquired forms. In the last decade, however, observations have accumulated which not only prove that hemolytic anemia may be acquired but which go far towards indicating some of the mechanisms involved.

The finding of an immune type of iso-hemolysin in three cases of acute hemolytic anemia led us to suspect that this was etiologically related to the excessive degree of hemolysis present.<sup>2</sup> This was borne out by experimental observations with hetero-hemolysins.<sup>3</sup> Guinea pig red cells, injected into rabbits, resulted in the development of anti-guinea pig hemolytic serum. When this serum was then injected in guinea-pigs, spherocytosis and acute hemolytic anemia developed. The observation was made that the small spherocytes were mature red cells and that the large red cells present were reticulocytes. This biphasic type of red cell population was considered to be distinctive for hemolytic anemia and the deduction was made that it was due to (a) hemolytic activity of hemolysin acting on mature red cells and producing spherocytosis, and (b) to regenerative activity on the part of the marrow, resulting in reticulocytosis. The disease hemolytic anemia was considered to be an active rather than a passive process, and not due to a marrow dysfunction.

Later, when the mechanisms for acute hemolytic disease of the newborn (erythroblastosis fetalis) were studied, it was apparent that Rh iso-antibody as developed by the Rh negative mother was responsible for injuring and thus destroying the Rh positive red cells of the fetus. Some Rh antibodies could be detected in salt solution, whereas in other cases, bovine albumin or plasma had to be used to demonstrate the agglutinin (blocking or univalent antibodies). With the use of another test developed later by Coombs, Mourant and Race,<sup>4</sup> it was apparent that antibody was firmly affixed (coated) to the red cell and could not be readily removed even with repeated washings of salt solution.

*Hetero-immunization* is rather readily understood: red cells from one species of animal (X-antigen) are injected into the circulation of another species: the second animal builds up a hetero-antibody (anti-X), this antibody can then injure the red cells of animals of the X-type and cause hemolytic anemia. Iso-immunization, too, seems fairly simple to comprehend, at least in its superficial aspects. Here, an individual lacking a specific factor (such as Rh) can be immunized by the red cells (antigen) of another individual of the same species, with the production of

an antibody When this antibody (anti-Rh) is then re-injected into the circulation of an individual with Rh positive red cells, antibody is adsorbed by the red cells. These become injured, i.e., spherocytic, or agglutinated, and are then removed from the circulation either by way of mechanical trauma or by splenic hemolysis.

In most cases of acquired hemolytic anemia other than in the type seen in the newborn, *auto*-immunization appears to be the central feature. The cause for this phenomenon, in which the individual's own red cells apparently develop antigenic qualities, thus producing an auto-antibody, is quite obscure. In any event, there can be little doubt that in practically all cases of acquired hemolytic anemia not due specifically to bacteria, parasites, chemicals or other definite factors, i.e., in the idiopathic cases, the plasma contains an *auto*-antibody which acts against the individual's own red cells. This is also an *iso*-antibody, since it reacts against all types of human red cells and can be detected both by the use of bovine albumin as a testing fluid and by the Coombs anti-globulin technic. Evidence is at hand indicating that this antibody causes a shortening of red cell survival time of foreign transfused red cells and of the individual's as well. Recent studies in our laboratory reveal a striking correlation between three tests: (1) *iso*- and auto-antibody as detected by the bovine albumin technic, (2) the anti-globulin test, and (3) the red cell survival time as determined by the Ashby technic. We find that auto-antibody is often higher in concentration than is *iso*-antibody.

Auto-immunization appears to be the prime factor in acquired hemolytic anemia. It occurs not only in the idiopathic cases, but in the symptomatic hemolytic anemia of such conditions as chronic lymphocytic leukemia. In the last three cases of this disorder we have observed, an auto-antibody of agglutinin type was demonstrable. As a result of auto-immunization, various types of antibodies may develop. It appears probable that antibody, affixing itself to the mature red cells of the affected individual, causes agglutination and other disturbances of the red cell membrane with resultant spherocytosis. The highest concentrations of antibody are associated with the greatest degrees of spherocytosis. These sensitized red cells are then acted upon either by complement, causing hemolysis, or more often are destroyed by the mechanical trauma of the circulation or selectively removed from the circulation by the spleen.

Splenectomy in acquired hemolytic anemia may or may not remove the largest single production center for auto-antibody formation, in any event, the chief spherocyte-destroying organ is removed. It is well to realize that splenectomy may be either wholly or partially ineffective, since continued production of antibody may occur. Future progress in acquired hemolytic anemia, which seems to be on the increase, lies in determining why auto-immunization develops and how it may be controlled.

WILLIAM DAMESHEK, M.D.

#### REFERENCES

- <sup>1</sup>CHAUFFARD M. A. Rev. gen. de clin. et de therap. 26: 199, 1912.
- <sup>2</sup>WIDAL F., ABRAMI P. AND BRULE M. Arch. des mal. du coeur 1: 193, 1905.
- <sup>3</sup>DAMESHEK W. AND SCHWARTZ S. O. New England J. Med. 218: 75, 1938.
- <sup>4</sup>— AND —. Am. J. M. Sc. 196: 769, 1938.
- <sup>5</sup>COOMBS R. R. A., MOURANT A. E. AND RACE R. R. Brit. J. Exp. Path. 24: 255, 1943.

# ABSTRACTS

JOSEPH E. ROSS, M D, *Editor*

ABSTRACTERS

HELEN W. BELDINO, M D, Winston Salem, N C  
SHEILA T. CALLENDER, M D, Oxford, England  
GEORGE E. CARTWRIGHT, M D, Salt Lake City  
ROGER C. CRAFTS, PH D, Boston  
CHARLES P. EMERSON, M D, Boston  
SOLOMON ESTREN, M D, New York  
CLEMENT A. FINCH, M D, Boston

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RAMON M. SUAREZ, M D, San Juan, Puerto Rico  
WM. N. VALENTINE, M D, Pacific Palisades, Cal  
JAN WALDENSTRÖM, M D, Uppsala, Sweden

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## ANEMIA

**INCIDENCE OF SICKLE CELL ANEMIA IN THE NEWBORN NEGRO INFANT** R. B. Scott, R. P. Crawford and M. Jenkins. From the Division of Pediatrics, Howard University School of Medicine and the Pediatric Service of Freedmen's Hospital, Washington, D. C. *Am J Dis Child* 75: 842-849, 1948

Two hundred and sixty-two newborn Negro infants were tested for sickling trait by the cover slip method on the first, third and fifth days of life. Positive reactions were obtained in nine or 3.4 per cent as compared to sixteen or 7.6 per cent in a group of 209 older children (one week to eleven years) similarly tested. Three of the newborns with sickling trait were examined at a subsequent date (seven to seven teen months) and the degree of sickling in each was found to be greater. No cases of sickle cell anemia were present in the group of newborns.

The factors which may contribute to the suppression of sickling trait in early infancy and to the lower incidence of active sickle cell disease in childhood are discussed. It is possible that further study using one of the more recently developed and sensitive tests or, as the authors suggest, by the alteration of the chemical composition of the blood of young infants, may furnish an answer to this latency phenomenon and perhaps even aid in determining which patients with the trait will develop anemia and why.

H. B. M.

**SICKLE CELL DISEASE STUDIED BY MEASURING THE SURVIVAL OF TRANSFUSED RED BLOOD CELLS** S. T. E. Callender, J. F. Nickel, C. V. Moore and E. O. Powell. From the Department of Internal Medicine, Washington University and Barnes Hospital, St. Louis, Mo. *J. Lab. & Clin. Med.* 34: 90-104, 1949

The Ashby technic of differential agglutination was used to study the survival of transfused cells in relation to sickle cell disease. Normal red cells transfused to patients with sickle cell anemia showed a normal survival time. Red cells from patients with sickle cell anemia transfused to normal recipient subjects showed a shortened average time of survival. Red cells from healthy donors with the sickle cell trait transfused to normal recipient subjects and to a patient with sickle cell anemia showed a normal survival time. The findings indicate that the defect in sickle cell anemia is inherent in the red blood cell. There is evidence to suggest that sickling is not a function of age of the cell but that the cells in sickle cell anemia vary constitutionally in their liability to sickle. The authors suggest that the difference between the anemia and the trait is qualitative and not simply one of degree.

G. E. C.

**DETECTION OF MILD TYPES OF MEDITERRANEAN (COOLEY'S) ANEMIA** C. H. Smith. From the New York Hospital and the Department of Pediatrics, Cornell University Medical College, New York, N. Y. *Am J Dis Child* 75: 505-527, 1948

A study of the blood of 181 persons in 47 families with Cooley's anemia revealed that asymptomatic individuals with Cooley's trait or the mild form of the disease were surprisingly common in New York City.

The diagnostic hematologic procedures employed and the characteristic blood changes are described in detail. The author stresses the importance of the hematocrit as a diagnostic procedure and of the findings of increased resistance of red cells to hemolysis in hypotonic solutions of sodium chloride, stippled red cells and the presence of hypochromic macrocytes despite the tendency to microcytosis.

The hereditary aspects of this disease remain a subject of controversy although perhaps the majority of investigators currently favor the hypothesis that the mild form results from heterozygosity of an inherited factor which when homozygous causes true Cooley's anemia. While the author's finding of characteristic hematologic abnormalities in healthy members of the families of affected children (both parents in every case of a severely anemic child) supports the view of a dominant hereditary factor, he does not consider this limited study conclusive evidence of such a genetic relationship. He does, however, stress the importance of the mild form of the disease and of the need for more widespread detection of these asymptomatic carriers who are a potential source of hereditary transmission of the overt form of the disease.

H. B. M.

**SURVIVAL OF TRANSFUSED ERYTHROCYTES FROM A DONOR WITH NOCTURNAL HAEMOGLOBINURIA** *J. V. Dineen and P. L. Mellison* From the Department of Clinical Pathology and the Medical Research Council Blood Transfusion Research Unit and Department of Obstetrics, Post-Graduate Medical School of London, England. *Lancet* 1: 397-392, 1949.

Red blood cells from a patient with paroxysmal nocturnal hemoglobinuria were transfused (1) to an adult with rheumatoid arthritis and (2) to an anemic premature infant who received at the same time some normal adult blood. Survival of both normal and abnormal erythrocytes was followed by the Ashby technique. The cells from nocturnal hemoglobinuria were destroyed more rapidly than normal especially in the adult recipient. This fitted in well with the *in vitro* observation that the baby's serum showed less hemolytic activity than the adult and 6 other infant sera tested against the patient's red blood cells.

Both the *in vitro* studies and the transfusion experiments suggest that the red cells in paroxysmal nocturnal hemoglobinuria vary in susceptibility to hemolysis. This variation does not appear to be related to age of the cells.

S. C.

**PAROXYSMAL COLD HAEMOGLOBINURIA OF NON-SYPHILITIC TYPE** *L. K. Mallet and M. D. Hickey* From the Mater Misericordiae Hospital, Dublin, Eire. *Lancet* 1: 387-390, 1949.

This is a full report of a well investigated case of paroxysmal cold hemoglobinuria associated with chronic hemolytic anemia and Raynaud phenomenon. There was no evidence of syphilis and the Donath-Landsteiner reaction was negative. A high titer cold agglutinin active over a wide thermal range was constantly present and the mechanical fragility of the patient's red cells was increased. He died after four years observation of urinary infection. Necropsy findings are reported.

S. C.

**HEMOLYSIS DURING TRANSURETHRAL PROSTATIC RESECTION** *C. L. Bjorn and L. F. Greene* From the Section on Urology, Mayo Foundation and Mayo Clinic, Rochester, Minn. *Surg., Gynec. & Obst.* 89: 369-375, 1949.

The incidence of hemoglobinemia following transurethral resection in a series of 100 cases selected at random was 56 per cent. The criterion used for hemoglobinemia in this study was a concentration of more than 25 mg. of hemoglobin per 100 cc. of plasma. As is pointed out, this concentration is somewhat higher than that used by other investigators who have reported a higher incidence of hemoglobinemia in similar although smaller case studies.

Excessive bleeding, the weight of the tissue removed, and the difficulty of resection appeared to be the significant surgical factors in inducing hemolysis, presumably by allowing much larger amounts of irrigating fluid (sterile water in this series) to wash into the circulation through venous sinuses. An analysis of postoperative reactions showed that gastrointestinal symptoms occurred twice as frequently in patients who had high concentrations of plasma hemoglobin as in those with no significant hemolysis. It is of interest that none of the patients experienced a postoperative chill, that the incidence of fever was greater in the group without hemolysis, and that there were no instances of postoperative oliguria other than one patient without significant hemoglobinemia in whom the oliguria was attributed to cardiac failure. Only six patients in this series had more than 500 mg. of hemoglobin per 100 cc. of plasma (one patient had 1,000 mg.). While levels of plasma hemoglobin higher than this may induce renal insufficiency, this

study of a statistically significant number of patients indicates that the concentration of hemoglobin-mia usually encountered during transurethral resection is not sufficient to be harmful

H B M

**MEGALOCYTIC ANAEMIAS** *J F Wilkinson* From the Department of Haematology Manchester Royal Infirmary England *Lancet* 1 49-55 291-296 336-340 1949

These Oliver Sharpey lectures given at the Royal College of Physicians London in March 1948 give a general review of megalocytic anemias colored by the author's own views and experience. As they were delivered a year ago there is no mention of work resulting from the discovery of vitamin B<sub>12</sub>. There is an initial summary of what was then known of the stomach principal extrinsic factor the liver principal and folic acid with emphasis on the work done by the author and his colleagues on the stomach factor. A discussion of the various types of megaloblastic anemia and the results of therapy follow. Finally the prognosis and incidence of cancer and other complications in pernicious anemia is discussed in relation to Wilkinson's own carefully observed series of 1 600 patients.

Some of the views expressed here of the relative inefficacy of some types of liver preparations particularly wartime and postwar British extracts have been challenged and the suggestion made that a lowered protein intake in the diet is a more relevant factor in suboptimal responses (*G E Shaw Lancet* 1 345-346 1949).

S C

**SEVERE ANAEMIA IN INDIAN SEPOYS (REFRACTORY TROPICAL MACROCYTIC ANAEMIA)** *R Passmore* From Indian Medical Service *Tr Roy Soc Trop Med & Hyg* 42 367-380 1949

One hundred and twenty seven cases of severe sometimes fatal and frequently refractory anemia were observed among sepoys serving in Assam and Eastern Bengal. Probable contributory factors were inadequate military hygiene recent malarial infection and malnutrition although evidence of these was not constantly present. Most of the blood examinations showed a macrocytic and either ortho- or hypochromic anemia. The bone marrow showed an increase in red cell precursors and an apparent shift to earlier forms but no megaloblastic change. The mortality was at least 38 per cent. Adequate diet control of infection and transfusions were the most effective therapeutic measures but even with these recovery was slow. Eighteen of 36 patients given liver injections showed a response which was possibly attributable to the liver but the general impression was that the anemia was not strikingly influenced by liver or yeast extracts.

Knowledge of the tropical macrocytic anemias is clearly far from complete. This group of cases does not seem to conform to the anemia described by Wills. The etiology is complex and the author suggests that long-standing nutritional defects and repeated malarial infections antedating military service were of prime etiologic importance.

S C

**OBSERVATIONS ON RELAPSES IN PERNICIOUS ANEMIA** *E Jones C C Tillman and W J Darby* From the Vanderbilt University Hospital Nashville Tenn *Ann Int Med* 30 374-380 1949

Liver extract was discontinued on 12 patients with pernicious anemia. Red count hemoglobin and determinations of fecal urobilinogen were made. Relapse was defined as a fall in red count on two successive measurements to more than two standard deviations below the average red count of the patient's during treatment. Six of the 12 patients failed to show hematologic relapse over a period of twenty six to twenty nine months. Eight to eighteen months were required to produce relapse in these patients. Of interest was the increase in urobilinogen above 350 Ehrlich units in some cases when the red count fell to between 2.5 and 3.5 million.

C A F

**INCIDENCE OF THE BLOOD GROUPS AND THE SECRETOR FACTOR IN PATIENTS WITH PERNICIOUS ANEMIA AND STOMACH CARCINOMA** *R P Ladenson S O Schwartz and A C Ivy* From the Hematology Laboratory and the Hektoen Institute for Medical Research of the Cook County Hospital and the Department of Clinical Research of the University of Illinois Chicago Illinois *Am J M Sc* 217 194-197 1949



This survey of the incidence of the blood groups (O A A<sub>1</sub> A<sub>2</sub> B AB A<sub>1</sub>B A B M N MN Rh<sub>+</sub> and Rh<sub>0</sub> negative) as well as the secretor and nonsecretor (gastric) attributes was undertaken in the attempt to determine whether any relationship existed between them and pernicious anemia and stomach carcinoma. No relationship was found between pernicious anemia and the blood groups or Rh negative oess. Patients with pernicious anemia secrete blood group specific substances in their saliva in the same proportion as normal individuals. The percentage of secretors and nonsecretors was approximately the same in pernicious anemia patients who showed gastric atrophy as it was in those who had a normal gastric mucosa. Patients with carcinoma of the stomach show an approximately normal distribution of blood groups and the secretor trait.

G.E.C.

THE TREATMENT OF SUBACUTE COMBINED DEGENERATION OF THE SPINAL CORD WITH VITAMIN B<sub>12</sub> T D Spies R E Stone S Karlus and T Aramburu From the Department of Nutrition and Metabolism Northwestern University at Hillman Hospital Birmingham Alabama South M. J. 41 1030-1031 1948

Three patients with pernicious anemia and acute neurologic manifestations of posterolateral sclerosis were treated with vitamin B<sub>12</sub>. Typically 25 micrograms of the material was given by injection every forty-eight hours for four injections. In all patients there was a rapid subjective and objective improvement beginning within two to five days after start of therapy.

S.E.

TENTATIVE APPRAISAL OF VITAMIN B<sub>12</sub> AS A THERAPEUTIC AGENT T D Spies R M Suarez, G G Lopez F Milanes R E Stone R L Toca T Aramburu and S Karlus From the Department of Nutrition and Metabolism Northwestern University at the Hillman Hospital Birmingham Alabama and at Calixto Garcia Hospital Habana Cuba and from School of Tropical Medicine San Juan Puerto Rico J A M A 139 521-525 1949

This clinical article reviews the effect of vitamin B<sub>12</sub> in a group of patients with macrocytic anemia. Four patients had nutritional macrocytic anemia, one had nontropical sprue, 11 had tropical sprue and 5 had pernicious anemia. In addition 14 patients with known pernicious anemia who also had posterolateral sclerosis were studied. It was found that, in all cases, the administration of vitamin B<sub>12</sub> (parenterally) was followed by rapid subjective and objective improvement. In the patients with anemia there was increase in strength, return of appetite, elimination of paresthesiae of the tongue and improvement in the character of the stools (in sprue). Reticulocytosis occurred and was followed by improvement in the levels of red cells, hemoglobin and leukocytes. In the patients with neurologic lesions there was alleviation of tingling, stiffness and numbness and remission of neurologic signs.

Details of dosage and management with B<sub>12</sub> are noted to require individual management in each particular case.

S.E.

EFFECTS OF FOLIC ACID ON THE ANEMIA INDUCED BY X IRRADIATION S P Stearns From the Biology Division of the Argonne National Laboratory Chicago Illinois Proc Soc Exper Biol & Med 89 515 521 1948

Pursuant to previous investigations (J Lab & Clin Med 32 1425 1947 Abst 37) indicating the lack of response to folic acid of the macrocytic anemia produced by the administration of radioactive strontium, the efficacy of this hemopoietic principle was tested in white rats receiving the approximate median lethal dose of total body x irradiation which is presumed to cause damage to viscera as well as to bone marrow. The results indicated that folic acid provided little or no stimulus to erythropoiesis following exposure to x radiation. The authors conclude, therefore, that the resultant bone marrow damage was attributable to direct injury and that irradiation damage to viscera involved in the elaboration of the anemia principle must have played a relatively unimportant role in the production of anemia.

C.P.E.

DIETARY EFFECTS ON ANEMIA PLUS HYPOPROTEINEMIA IN DOGS F S Robbert Robbins and G H Whitely From the Department of Pathology the University of Rochester School of Medicine and Dentistry Rochester New York J Exper Med 89 339-368 1949

In order to study the production of hemoglobin and plasma protein by various specific food proteins dogs were first depleted of hemoglobin and plasma proteins by frequent blood removal and a nonprotein diet containing all other dietary essentials

I Some Proteins Further the Production of Hemoglobin and Others Plasma Protein Production (pp 339-358)

Although there was a satisfactory production of total blood protein with the various egg fractions fresh and processed fresh and processed meat fresh beef heart and canned salmon muscle, certain quantitative and qualitative differences were noted. In general a meat diet produced a greater amount of new blood protein than did the several egg products. Also on a meat diet the hemoglobin production greatly exceeded the plasma protein production whereas the egg protein diets favored the production of plasma proteins. More specifically the hemoglobin production with fresh beef muscle was three or four times that of plasma protein and the output of total blood protein with fresh or processed beef muscle twice that obtained with the egg diets. Beef heart and salmon muscle produced a pattern similar to beef muscle except that the total blood protein output was less. Processed egg albumin was the only egg product not well utilized.

II The Findings with Milk Products Wheat and Peanut Flours as Compared with Liver (pp 359-368)

Liver was used as a control in these experiments as it gives maximum amounts of newly formed blood protein with a hemoglobin production of approximately three times that of plasma protein. Casein was found to compare favorably with liver and meat. Lactalbumin was not as effective as casein but like the egg proteins it favored plasma protein production. Peanut flour gave a poor response. While the response to wheat gluten was better than that with peanut flour its unpalatableness presented difficulties in experimental control.

These two papers are an extension of previously reported studies on body and blood proteins. Further work on this subject is being carried out with radioactive isotopes to determine more accurately the exchange which can take place between body and circulating proteins in protein-depleted dogs.

H B M

THE EFFECTS OF SULFONAMIDES ON AMBLYSTOMA EMBRYOS WITH PARTICULAR REFERENCE TO BLOOD DEVELOPMENT *W. M. Copenhaber and S. R. Detweiler* From the Department of Anatomy College of Physicians and Surgeons Columbia University, New York N. Y. *J. Exper. Zool.* 109 239-257 1948

Sulfonamides have been used successfully in reducing mortality rates following surgical procedures on Amblystoma embryos. Since some animals showed toxic effects the present investigation was undertaken to study the effect of different concentrations of sulfadiazine and sulfanilamide on embryos at the blastula, gastrula and tail bud stage of development. The range of drug concentration in spring water was 0.12 to 2.0 per cent. Anemia was more frequent and more pronounced in sulfanilamide treated animals. Splenic development was markedly suppressed. Granulopoiesis in the subcapsular region of the liver was apparently unaffected. The anemia seemed to be a combination of aplastic and hemolytic types. Because of the hematologic response of these animals they may be useful for testing the effects of other drugs.

O P J

FACTORS INFLUENCING THE BLOOD PICTURE OF THE NEWBORN. STUDIES ON SINUS BLOOD ON THE FIRST AND THIRD DAYS *Q. B. DeMarsh, H. L. Alt and W. F. Windle* From the Department of Medicine and Anatomy Northwestern University Medical School and the Cook County Hospital Chicago Ill. *Am. J. Dis. Child.* 75 860-871 1948

In order to determine the factors which may influence the blood picture in the newborn and which have mainly accounted for the present lack of standard blood values the authors studied the effects of early and late clamping of the umbilical cord by heel punctures and blood volume determinations (see *J. A. M. A.* 116 2568 1941 and *Am. J. Dis. Child.* 63 1123 1942) and in the present report by observations on sinus blood.

The most important cause of variation in blood values is the time of clamping of the cord after delivery. When clamping is delayed until the placenta has separated approximately 108 cc of placental blood otherwise lost by immediate cord clamping is added to the infant's circulation, a rapid adjustment of plasma volume occurs, and the infant's blood volume is increased by the volume of these additional

red cells. Significantly higher values for hemoglobin, red cell count and hematocrit were obtained when clamping was delayed. For example, the mean value of hemoglobin in sinus blood on the first day was 20.6 Gm per cent compared to 16.4 Gm per cent when the cord was clamped immediately on delivery.

Other variable factors were the source of blood (blood levels were higher with capillary than with sinus blood) and, of less significance, the time after birth at which the blood sample was taken.

That increased erythropoiesis is prolonged, probably as a compensatory mechanism, in the group deprived of the additional placental blood was indicated by a comparison of the reticulocyte counts made on the third day in the two groups.

H.B.M.

## ERYTHROCYTE FRAGILITY

AN IMPROVED METHOD FOR THE DETECTION OF OSMOTIC ABNORMALITIES OF ERYTHROCYTES. *M. H. Jaishankar, D. R. Stewart, W. J. Brown and L. J. Kammelman*. From the Department of Physiology, University of Pennsylvania School of Medicine, Philadelphia, Pa. *Am. J. M. Sc.* 217: 47-52, 1949.

In order to avoid certain sources of error in the usual clinical fragility test with hypotonic salt solutions, as well as to shorten the time and reduce the number of solutions required for a test, an alternative procedure is suggested in which a continuous hemolysis curve is obtained, either with or without photographic recording, in a solution containing a penetrating solute such as thionin or glycerol.

Using this method the hemolysis curves are similar to those obtained with hypotonic salt solutions, but small individual peculiarities are brought out.

G.E.C.

THE OSMOTIC RESISTANCE OF HUMAN ERYTHROCYTES IN NORMAL, CARRIER AND ANEMIC STATES WITH SPECIAL REFERENCE TO CHANGES DUE TO AGE, RACE, SICKLE-CELL ANEMIA, MEDITERRANEAN (COOLBY) ANEMIA AND CONGENITAL HEMOLYTIC ICTERUS. *B. Dickstein, W. E. Landmesser, Jr., W. E. Lee, T. H. Wilson and I. J. Wolman*. From the Children's Hospital of Philadelphia and the Departments of Pediatrics and Physiology, University of Pennsylvania School of Medicine, Philadelphia, Pa. *Am. J. M. Sc.* 217: 53-61, 1949.

Utilizing a reproducible method, photographic records of the hemolysis of erythrocytes from 170 normal persons and 90 anemic individuals have been obtained. In white subjects the osmotic resistance of the erythrocytes in the 0 to 10 year age group was found to be greater than that of normal adults. A comparable age difference was not found in a limited number of Negroes. The average osmotic resistance of the erythrocytes of the normal Negro was greater than that of the normal white in the corresponding age group. The erythrocytes of children with sickle cell anemia were more resistant than those of normal Negro children. The average for individuals with the sickle cell trait was between that for sickle cell anemia and that for the normal, but with considerable overlapping in both directions. In both Mediterranean anemia and its carrier state the blood was markedly more resistant than that of the normal controls, the carrier showing a resistance as great as that of the anemic individual.

G.E.C.

THE TONICITY VOLUME RELATIONS FOR SYSTEMS CONTAINING HUMAN RED CELLS AND THE CHLORIDES OF MONOVALENT CATIONS. *E. Pender*. From The Nassau Hospital, Mineola, Long Island, N. Y. *J. Gen. Physiol.* 32: 391-398, 1949.

It has been known that when red cells are suspended in solutions of different ionic composition but of the same depression of the freezing point, that there are discrepancies in the relation between tonicity and volume. Chlorides of monovalent cations obtained from two different sources were prepared in a 0.172 M solution in water. The salts used were LiCl, NaCl, KCl, RbCl and CsCl. Red cells were most fragile in LiCl and least fragile in NaCl. The  $K^+$  losses in LiCl were so small and slow that they could not be counted for the increased fragility. It has been pointed out that there are differences in the molarity of solutions which are isotonic with plasma.

O.P.J.

THE TONICITY VOLUME RELATIONS FOR HUMAN RED CELLS SUBJECTED TO THE ACTION OF HEAT WITH SPECIAL REFERENCE TO PROLYTIC K LOSS. *E. Ponder* From The Navan Hospital, Min-ola, Long Island N. Y. *J. Gen. Physiol.* 3: 399-405, 1949.

It has been thought that heating red cells for short periods between 49.6°C and 50.6°C had small effect on the swelling which occurs in hypotonic media of different tonicity. Suspensions of washed red cells in NaCl were heated for two minutes at 45°C and 50°C and then allowed to cool to 25°C. After an hour at this temperature, samples were obtained for the determination of cell volume and the extent of hemolysis. At 45°C, heated and unheated cells behave equally well as osmometers, but those heated at 50°C have an impaired ability to swell in hypotonic solutions. Heated cells lyse in higher tonicities than unheated ones. Some of these findings may be accounted for by the large K losses and K-Na exchange. O.P.J.

## IMMUNOHEMATOLOGY AND TRANSFUSION

ANÉMIE HÉMOLYTIQUE AIGUE ÉRYTHROBLASTIQUE TRÈS TARDIVE (VERY LATE ACUTE ERYTHROBLASTIC HEMOLYTIC ANEMIA). *M. Kaplan, M. Bessis, F. Barratt, P. Dilhailar, J. C. Caine*. *Arch. Franç. Ped.* 5, No. 6, 1948.

The third child of a family with an Rh negative mother and Rh positive father was affected by a hemolytic anemia which did not appear until the seventh week after birth. Antibodies were found at the examination 25 days after delivery (1:1 in saline, 1:8 in albumin medium). The Coombs test was positive.

The second child had had a similar anemia when he was 6 weeks old, probably of the same nature.

Between the first and the second pregnancy, the mother received a transfusion with Rh positive blood which may have increased her iso-immunization.

The child was fed with cow milk, and thus the maternal antibodies could not have been given by any other route than the transplacental. It is difficult to decide whether the maternal antibodies fixed themselves on the infant red cells a long time after birth, or whether they were fixed early and destroyed the cells only after a long interval.

The very severe anemia of this third child was treated with Rh positive transfusions, which was believed to be preferable to Rh negative blood, since there were no more maternal antibodies in the infant's circulation at this time.

J.P.S.

USE OF BLOOD DONORS WITH POSITIVE SEROLOGIC TESTS FOR SYPHILIS WITH A NOTE ON THE DISAPPEARANCE OF PASSIVELY TRANSFERRED REAGIN. *M. M. Raistch, T. W. Farmer, and B. Davis*. From the Departments of Surgery and Medicine, the Johns Hopkins University and Hospital, Baltimore, Md. *J. Clin. Investigation* 28: 18-23, 1949.

Sixteen patients with negative serologic tests for syphilis were studied after receiving injections of plasma from blood donors with positive STS. The period of storage before separation of plasma from the luteal donor bloods varied from one to ten days, and the intervals between freezing and thawing of the plasma ranged from two weeks to two months. In all instances a positive STS was acquired by the recipient; the initial titer of which represented the dilution to the recipient's blood volume of the reagent contained in the injected plasma. Reversion of the tests to negative occurred in all instances within a period of 20 days. It is concluded that the blood of donors with syphilis should be acceptable for use in any blood bank with a plasma program, inasmuch as infectivity of the material is abolished by freezing or by storage for a minimum of four days in the refrigerator.

C.P.E.

THE VERY RARE RH GENOTYPE  $R_{YK}$  (CoE/CdE) IN A CASE OF ERYTHROBLASTOSIS FOETALIS. *C. van den Bosch*. From the Department of Pathology, University of Louvain, Belgium. *Nature* London 162: 781, 1948.

In a case of erythroblastosis foetalis, the mother's blood group was O(MNP) and the cells were agglutinated by anti-C, anti-E but not by anti-D. Her serum contained complete and incomplete anti-D. She thus belonged to the very rare allelic combination CdE (Wiener's  $r_{YK}$ ) predicted by Fisher in 1944.

Detailed study of the family made it possible to identify CdE as an inherited combination on one chromosome. This discovery brings additional support to Fisher's already very well founded theory. STC.

**A HEMOLYSIN ASSOCIATED WITH THE MUMPS VIRUS** *H R Morgan, J F Enders and P F Wagley* From the Research Division of Infectious Diseases of the Children's Hospital, Children's Medical Center and the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard) Boston City Hospital, Harvard Medical School, Boston, Massachusetts. *J. Exper. Med.* **81**: 503-514, 1948.

The observations made on an hemolysin found in the amniotic and allantoic fluids of chick embryos infected with the virus of mumps are presented. Chicken, sheep and human erythrocytes were all susceptible to hemolysis although those of man were less affected.

It appears evident from the following observations that this hemolytic activity is due to a specific product of the mumps virus: (1) a similar hemolysin could not be demonstrated in normal egg fluids or in those infected with two strains of influenza virus; and (2) the hemolysin could be inhibited by mumps convalescent sera from man and monkey but only slightly by normal monkey serum taken during the early stage of the disease in man.

The authors have inferred from their study of the effect of heat, temperature and time of incubation and of pH on the hemolysin that this hemolytic activity is not identical with the hemagglutinative property of the infected fluid. That some as yet undefined relationship exists between these two factors, however, is indicated by the similarity of their behavior in respect to adsorption on and elution from chicken red blood cells and their inhibition by specific immune serum. Attention is also drawn to the enzyme-like behavior of the hemolysin.

H.B.M.

**GENETIC TRANSMISSION OF TWO RARE BLOOD GROUP GENES** *A. S. Wiener* Jewish Hospital, Brooklyn, N. Y. *Nature* (London) **162**: 735, 1948.

This note records the phenotypes and genotypes of four families, three of whom show transmission of the gene  $R^s$  and one of the extremely rare  $r^Y$ .

STC.

**HEMOLYTIC ANEMIA ASSOCIATED WITH ATYPICAL HEMAGGLUTININS** *W. J. Kabus and P. F. Wagley* From the Department of Medicine, School of Medicine, Johns Hopkins University and Baltimore Rh Typing Laboratory, Baltimore, Md. *Ann. Int. Med.* **30**: 408-423, 1949.

A very interesting case is reported showing intravascular thrombosis and atypical hemagglutinins in high titer. In addition to cold hemagglutination, a warm hemagglutinin was demonstrated which reacted at 37 degrees with the patient's cells and with 63 per cent of bloods compatible for  $A_1$ ,  $A_2$ ,  $O$ ,  $M$ ,  $N$ ,  $Rh$  and  $Hr$ . The nature of these agglutinins and their possible role in hemolytic anemia and intravascular thromboses are discussed in competent and interesting fashion.

C.A.F.

**CLINICAL USE OF BLOOD DERIVATIVES** *C. A. Janeway* From the Department of Pediatrics, Harvard Medical School and the Children's Medical Center, Boston, Massachusetts. *J. A. M. A.* **138**: 859-865, 1948.

This survey of the field of blood derivatives summarizes the advances of therapeutic knowledge of these substances especially during the past ten years. In brief, the following points are covered:

1. **Blood Cells** White cells and platelets can as yet not be satisfactorily separated and preserved; red cells can. Red cells resuspended in saline to a hematocrit of 65 per cent constitute an ideal treatment for certain patients with anemia, for (a) it is possible thus to supply more hemoglobin with less loading of the circulation, and (b) the removed plasma may be reserved for use for other patients. Hemoglobin itself has been prepared from red cell fractions for possible use in traumatic shock; experimentally, its high oxygen-carrying capacity combined with its high osmotic activity make it theoretically excellent for shock treatment, but actually the injection of pure hemoglobin is often followed by depression of renal function, so that its clinical use has had to be cautious to the extreme. Globin itself may be used as a substitute for plasma proteins.

2. **Plasma** Whole plasma is, of course, used widely in the treatment of shock. The occurrence of homologous serum hepatitis after the administration of plasma, however, has been a drawback. Statistics are presented as to the incidence of this disorder under various conditions; single transfusion of blood

or serum (hepatitis rare) use of fraction I from pooled plasma (hepatitis in 10 per cent of subjects) use of pooled plasma or serum (hepatitis in 4 to 7 per cent of recipients) Methods of preventing this hepatitis are mentioned of these only sterilization of the plasma seems potentially practicable The author suggests the use of pooled plasma only if neither blood nor serum albumin are available

3 Plasma Fractions Discussed are *factors important in coagulation* (fibrin, thrombin fibrinogen anti hemophilic globulin) *blood grouping globulins disease antibodies* (gamma globulin for measles hepatitis, mumps) and *albumin* All these substances have already found widespread clinical use for their particular qualities

The article is not meant to be all inclusive but covers the salient material in a brief salient manner  
S E

## IRON METABOLISM

CHEMICAL CLINICAL AND IMMUNOLOGICAL STUDIES ON THE PRODUCTS OF HUMAN PLASMA FRACTIONATION

XXXIX THE ANEMIA OF INFECTION STUDIES ON THE IRON BINDING CAPACITY OF SERUM *G E Carlwright and M M Wintrobe* From the Department of Medicine, University of Utah School of Medicine Salt Lake City Utah J Clin Investigation 28 86-98 1949

From determinations of the serum iron concentrations and unsaturated iron binding capacity of serum from which the total iron binding capacity and per cent of iron saturation of serum were calculated, the authors conclude that the hypoferrernia accompanying infections is not the result of a reduction in the iron binding capacity of serum but must depend upon some other factor The total iron binding capacity of serum in 30 normal individuals averaged approximately 360 gamma per cent the iron binding protein being approximately 35 ( $\pm 6$ ) per cent saturated with iron In 13 patients with chronic infection in 2 dogs with sterile abscesses and another with an acute infection the total iron binding capacity was significantly reduced but the reduction in serum iron was proportionately greater, with the result that the per cent saturation was lowered

Measurements of serum iron concentration following intravenous iron injections indicated that the concentration peaks were limited by the capacity of the serum to bind iron, and when the total iron binding capacity of the serum was exceeded the unbound iron rapidly left the blood stream with the concomitant development of toxic symptoms The administration of metal-combining globulin (fraction IV 7) to 2 patients with chronic infection resulted in a temporary increase to normal in the total serum iron binding capacity Subsequent intravenous injections of iron resulted in a greater initial five minute rise in the serum iron concentration than had previously been noted but the rate of iron disappearance from the serum was not significantly affected Moreover, the temporary artificial increase in iron binding capacity was not followed by a detectable mobilization of iron in the blood

C P E

CHEMICAL CLINICAL AND IMMUNOLOGICAL STUDIES ON THE PRODUCTS OF HUMAN PLASMA FRACTIONATION

XXXVIII SERUM IRON TRANSPORT MEASUREMENT OF IRON BINDING CAPACITY OF SERUM IN MAN *C E Rath and C A Finch* From the Department of Medicine Harvard Medical School and the Medical Clinic Peter Bent Brigham Hospital Boston Mass J Clin Investigation 28 79-85 1949

A protein constituent of plasma a beta 1 globulin with the capacity to bind metal ions particularly iron copper and zinc (Science 104 340 1946) has been measured in normal and pathologic sera by these authors who performed concomitant measurements of the serum iron concentration and computed the unsaturated iron binding capacity as well as total iron binding capacity of these sera In a group of 30 normal subjects the serum iron concentration averaged 100 gamma iron binding capacity 200 gamma and total capacity 300 gamma per 100 cc of serum the circulating iron binding protein was on the average 34 per cent saturated with iron

In cases of iron deficiency whereas the serum iron concentration was lowered both the unsaturated iron binding capacity and the total iron carrying capacity of the serum were elevated In the presence of infection not only the serum iron but also the iron-binding capacity and total capacity were reduced In a variety of debilitating conditions with associated hypoproteinemia there was a reduction in the iron binding capacity of the serum and no elevation of the latter was observed even when iron deficiency was present as an additional complication No deviation from the normal was found in pregnant women Elevated values for serum iron and percentage saturation of the iron binding protein were found in refractory anemia, pernicious anemia hemochromatosis transfusion hemosiderosis and liver disease

Human plasma fraction IV- $\gamma$  binding iron in the proportion of 1 ml per ml of prot in was injected intravenously into 22 individuals in amounts of 2.5-5.0 grams the injection times ranging from 15 to 30 minutes. The injections which were without incident were followed by a rise in serum iron which reached a peak 12 to 24 hours after injection and subsided over a period of 2 to 6 days excepting in cases of hemosiderosis and hemochromatosis in whom a more sustained elevation occurred.

The evidence indicated that the body iron is completely protein-bound which perhaps explains the lack of a physiologic mechanism for iron excretion. Excepting in terminal hemochromatotic patients in whom some of the excessive serum iron is apparently bound to other proteins, the serum iron is found exclusively in combination with the beta<sub>2</sub> globulin designated as the iron binding protein (J Clin Investigation 28: 73, 1949). The elevations in iron binding protein observed in cases of iron deficiency may be responsible for the enhanced iron absorption in this condition, but the relation of iron-binding capacity to the facility and rate of iron absorption remains to be established. The finding of increased iron saturation implying the co-existence of bone marrow block, iron excess and severe liver disease is of great value in the differential diagnosis of hemochromatosis and simple cirrhosis.

CPE.

A STUDY OF HISTOCHEMICAL IRON USING TRACER METHODS. Y. M. Endicott, T. Gillman, G. Breiter, A. T. Ness, F. A. Clarke and E. P. Adamik. From the Pathology Laboratory, Experimental Biology and Medicine Institute, National Institutes of Health, Bethesda, Md. J. Lab. & Clin. Med. 34: 414, 1949.

Combined histochemical, radioautographic and tracer methods were used to study the absorption and distribution of single test meals of radioiron in guinea pigs, rats and one dog. The iron demonstrated histochemically in the duodenal mucosa, the mesenteric and cervical lymph nodes, liver and spleen was derived largely from sources other than the single test meal. This visible iron did not undergo well defined cyclic changes after a single test meal. It accumulated over a period of days or weeks of continued intake of a diet containing considerable iron. It behaved more like storage iron than iron in transport. The visible granular iron in the duodenal epithelium exerted no demonstrable effect on the amount of iron absorbed, and it did not appear to be a morphologic expression of mucosal block. Most of the radioiron of the test meal traversed the duodenal epithelium rapidly. In one dog it was transported from the intestine via the portal vein, only insignificant amounts being found in the thoracic duct lymph. There was no evidence to indicate that the reticulo-endothelial system participated directly in the absorption of iron from the intestine or transport of the absorbed iron from the intestine to the liver, blood and other organs and tissues.

GEC.

## POLYCYTHEMIA VERA

BLOOD OXYGEN STUDIES IN PATIENTS WITH POLYCYTHEMIA AND IN NORMAL SUBJECTS. L. P. Wasserman, R. L. Dobson and J. H. Laurence. From the Division of Medical Physics and Division of Medicine, University of California, Berkeley, Calif. J. Clin. Investigation 28: 60-65, 1949.

Determinations of the arterial blood oxygen saturation were made in 74 individuals of whom 48 were cases of polycythemia vera. The data presented indicated that the degree of arterial oxygen saturation was within the limits of normal in resting polycythemic subjects, normal values being found in patients with hematocrits as high as 81 per cent. The authors suggest that low oxygen saturation figures reported in the literature may have been attributable to technical errors inherent in gasometric measurements, which may have resulted from failure to conduct tests promptly after the blood samples were obtained. The erroneously low oxygen saturation values may depend on the fact that inactive hemoglobin (J. Biol. Chem. 138: 563, 1941) as well as carboxyhemoglobin are converted in vitro to normal reactive hemoglobin with resultant increase in the apparent oxygen capacity.

CPE.

## LEUKOCYTES

PRIMARY SPLENIC NEUTROPENIA. M. S. Sacks and T. N. Carey. From the Department of Medicine, University of Maryland School of Medicine and College of Physicians and Surgeons, Baltimore. South. M. J. 41: 922-925, 1948.

This is a case presentation of a 56 year old woman who at the age of 55 developed fatigue followed by otitis followed by furunculosis. Examination revealed hepatosplenomegaly. There was a slight anemia but the striking finding in the blood was a leukopenia (1100) with granulocytopenia (19 per cent neutrophils). The bone marrow was somewhat hypocellular and was considered to show decreased number of granulocytes with a shift to the left. Ultimately splenectomy was undertaken. The spleen weighed 2,200 grams and showed congestion. Following operation the white count rose to normal levels (e.g. 10,700) with normal granulocyte counts (e.g. 69 per cent). The blood count remained normal during the following two years when the patient died of pulmonary atelectasis and apparent hepatic disease. The liver at autopsy showed congestion.

Unfortunately meager data are presented and the exact mechanisms of neutropenia cannot easily be interpreted. It seems likely however that the case is one of the generic hypersplenic neutropenia group relieved by splenectomy.

S E

**PRIMARY SPLENIC NEUTROPENIA. A SPECIFIC INDICATION FOR SPLENECTOMY.** L. T. Palumbo. From the Department of Surgery, Veterans Administration Hospital, Des Moines, Iowa. *Ann Surg* 129: 131-136, 1949.

A patient with a marked leukopenia (2,500 per cu. mm.) marked splenomegaly without anemia or thrombocytopenia was splenectomized with complete alleviation of the leukopenia. This patient was followed 56 days postoperatively. No specific pathology was found in the spleen. The bone marrow as determined by sternal puncture was cellular. The author concludes that this was a case of primary splenic neutropenia and that the disease results from splenic dysfunction as a result of selective destructive action of the reticulo-endothelial cells of the spleen. It is to be regretted that this patient was followed for such a short period of time.

G E C

**CHRONIC CYCLICAL GRANULOPENIA.** B. Barling. *Proc Roy Soc Med* 41: 653-4, 1948.

This note discusses the occurrence over a period of twenty years of periodic ulceration of the mucous membranes in association with leukopenia and neutropenia. The patient, a woman, developed recurrent ulcers of the tongue, mucosa of the cheek and skin of the angles of the mouth approximately every four weeks from the age of 12 on. During pregnancy at the age of 30 additional lesions at the vulva occurred. At the age of 31 ulcers developed also at the lower leg and resisted healing.

Physical examination except for the ulcerations and their scars was regularly negative. Spleen, liver and lymph nodes were not palpable. The red cell count, hemoglobin and sternal marrow punctures were normal. The persistent abnormality was leukopenia which was due to neutropenia. Typical counts ranged from a total white count of 1,300 to 4,800 with granulocytes from 300 to 3,000 per cu. mm. (normally granulocytes range 3,000 to 6,000 per cu. mm.). Treatment with liver, hog's stomach, nicotinic acid, pyridoxin, pentanucleotide, nucleic acid, yellow bone marrow extract and iron had no effects either on the blood count or the lesions.

The etiology for this type of abnormality has never been explained. The ulcerations are considered the result of lowered resistance due to granulocytopenia. Treatment is universally ineffective. (See H. A. Reimann, *J. A. M. A.* 136: 238-244, 1948.)

S E

**CHRONIC GRANULOPENIA.** B. Barling. *Proc Roy Soc Med* 41: 654-5, 1948.

This case report concerns chronic neutropenia associated with splenomegaly in a 17 year old girl. These abnormalities were first discovered at the age of 15 during the course of investigation of an acute gastroenteritis. In the following six months she required hospitalization for persistent and recurrent fever, pallor and infections including a severe bout of pneumonia. The positive physical findings included a just palpable liver and an easily palpable spleen; there was no lymphadenopathy. Anemia was present (R.B.C. 1.54 million, hemoglobin 25 per cent), platelets were normal and the white count was low (e.g. 3,200 with 11 per cent granulocytes). A bone marrow puncture was normal.

After blood transfusion and recovery from pneumonia the patient remained in good health and developed normally. The spleen however continued to increase steadily in size and the white count and granulocyte count were regularly low. The question of splenectomy was tabled because of a negative adrenalin test.

This case of course is different from the cyclic neutropenia without anemia and without splenomegaly.



described by Barling (Proc Roy Soc Med 41 633-4 1948 see preceding abstract) and others and perhaps corresponds better to splenic neutropenia or splenic neutropenia with anemia (Doan and Wiseman, Dameshek) in which splenectomy may be expected to give beneficial results

SE

**MECHANISMS OF LEUKOPENIA WITH INFLAMMATION. AN ADDITIONAL LEUKOPENIC FACTOR FOUND IN ALKALINE EXUDATES.** V Menkin. From Chase Foundation for Cancer Research Temple University School of Medicine Philadelphia Pa Arch Path 46 145-158 1948

Exudative material withdrawn from the pleural cavities of dogs injected with turpentine are usually alkaline and they contain an extractable leukocytosis promoting substance. When this material is allowed to age for several months some of its properties change by becoming insoluble in isotonic saline. The leukocytosis-promoting substance is in the supernatant while a leukopenic component is in the insoluble residue. This leukopenic component differs from the one found in acid exudates by being inactivated by incomplete hydrolysis with tenth normal hydrochloric acid. However it appears that both of these factors exist in combination in fresh exudates and therefore help to explain the mechanism of leukopenia with inflammation.

OPJ

**DESTIN DES GRANULOCYTES TRANSFUSÉS (FATE OF TRANSFUSED GRANULOCYTES)** Bernard Dreyfus Sang 19 570-574 1948

A transfusion of 600 cc of myeloid leukemic blood containing 250 000 granulocytes by cubic millimeter was done in each of two recipients affected with subacute hemocytoblastic and lymphoblastic leukemias.

In both the increase was very short and the survival of the white cells was under 30 minutes. The blood examination shows in this initial period many forms of destruction. Thus the cell's destruction seems to be intravascular and not intracellular as has been said. The lysed cells also disappear very quickly from the blood stream and this explains the difficulty encountered in observing the phenomenon.

The total white cell count was lower 2 hours and 24 hours after the transfusion than it was before (31 500 against 50 000 in the first case 41 400 against 52 000 in the second case). This suggests that some of the white cells of the recipient are destroyed in the first hours following transfusion.

These results are to be compared with those of Minor and Isaacs (1925) who found a similar reduction of the injected white cells injecting lymphoid leukemic cells to a patient with lymphosarcoma but injecting only 450 cc of blood containing 89 200 white cells per cubic millimeter. They found a very slight modification in the white count and they did not find any lysed cells.

Dreyfus's conclusions are that no substitutive effect is to be found for white cells in blood transfusion, and that all increase in white cell count found after blood transfusion expresses only the regeneration capacity of the recipient.

JPS

**A MACROPHAGE PROMOTING-FACTOR (MPF) IN THE BLOOD OF RABBITS.** C M Pomeroy W Jacobson and M F Orr. From the Department of Anatomy University of Texas Medical Branch Galveston Texas Am J Anat 84 1-19 1949

When embryonic chick spleen fragments were implanted it was observed that in some instances cultures containing 25 per cent normal rabbit serum would produce great numbers of phagocytic cells which ingested myelocyte debris. Experiments were undertaken to determine the occurrence and nature of this macrophage promoting factor (MPF). Control hanging drop preparations were grown in a medium of 50 per cent fowl plasma 25 per cent embryonic juice and 25 per cent Tyrode's solution. In the test preparations heterologous sera or resuspended fractions of sera were substituted for the Tyrode component. In cultures containing MPF the area of outwandering cells was markedly reduced, myelocytes were not at the periphery and many macrophages were at the peripheral zone. This factor was not present in all animals and it even varied within a given animal over a period of months. Oddly enough MPF was present only in the species known to have the Forssman antigen. Properties of the MPF are: it is thermostable and resists freezing-drying; it is insoluble in absolute alcohol or acetone but soluble in Tyrode after precipitation in 1/3 saturated ammonium sulfate. It does not seem to be identical with any of the factors in inflammatory exudates as reported by Menkin.

OPJ

## BLOOD COAGULATION

PROTHROMBIN CONVERSION FACTOR OF DICUMAROL PLASMA *C A Owen and J L Bollman* From the Division of Experimental Medicine, Mayo Foundation, Rochester Minnesota Proc Soc Exp Biol & Med 67 231-234 1948

Data obtained from experiments on dicumarolized dogs suggests that the hemorrhagic diathesis produced by dicumarol is attributable not alone to a disappearance of prothrombin but also to the loss of a factor, the function of which is to facilitate the conversion of prothrombin to thrombin. Variations in the concentration of this conversion factor present in plasma serum, or serum pseudo-globulin may explain the familiar discrepancies in the results of one and two-stage methods of estimating prothrombin activity. It may also account for the therapeutic efficacy of serum in the treatment of cattle with sweet clover disease, a phenomenon otherwise difficult to explain.

C P E

ACTION DE LA PHENYL INDANE DIONE SUR LE TAUX DE LA PROTHROMBINE I ETUDE EXPERIMENTALE SUR LE LAPIN *J P Soulier and J Guiguen* II UTILISATION EN CLINIQUE HUMAINE. (EFFECT OF PHENYL INDANE DIONE ON PROTHROMBIN LEVELS I EXPERIMENTAL STUDIES ON THE RABBIT II USE ON HUMANS) *J Guiguen and J P Soulier* Rev Hemat 3 180-195 1948

In the first series of experiments using 16 rabbits the authors found that phenyl indane-dione (PID) had a very marked effect on prothrombin level. Doses of 10 to 20 milligrams per kilo produced a decrease of prothrombin to a level of 30 to 40 per cent this effect being reached before the eighteenth hour. There was no modification of platelets clot retraction or fibrinogen level. Higher dosage did not produce greater hypoprothrombinemia and the authors did not find any hemorrhages even with a dosage ten times the standard dosage. The lethal dose was well over 600 mg/kilo which gave a very high safety margin. Histologic examinations of the rabbits given very high doses of PID (under 400 mg/kilo) did not show histologic injuries.

The PID was used in the prevention of thrombosis in 43 women after pregnancy. In all these cases doses of 10 to 20 mg/kilo yielded a very constant decrease of prothrombin level. The decrease began earlier than with dicumarol about the twelfth hour and the full effect was obtained between the twenty-fourth and the forty-eighth hour which is a 30 to 40 per cent level. Return to a normal level was quite constant and 100 per cent prothrombin was reached by about the ninety-sixth hour.

This constancy in the chronology is very different from that observed with dicumarol. Individual susceptibility to the drug seems also to be less important than in the case of dicumarol.

In 2 cases the PID was given to patients with known thrombophlebitis (every 3 days 10 mg/kilo). This dose was effective in controlling the prothrombin level around 30 per cent. The patients' state was, in both cases favorably affected. In the 41 cases where the drug was given prophylactically no phlebitis was observed.

In contrast with these advantages the complete inactivity of vitamin K<sub>3</sub> even in huge doses and even when given prior to the administration of the PID must be emphasized. But this fact is perhaps of minor importance since in no case was hemorrhage or hypoprothrombinemia of less than 10 per cent observed.

J P S

## LEUKOCYTES, LEUKEMIA AND LYMPHOMA

LA PLASMOCYTOSE CANCÉREUSE (THE PLASMA CELL REACTION OF CANCER) *G Marchal and L Mallet* Sang 19 457 1948

Myelocytosis eosinophilia megakaryocytosis are common bone marrow reactions in case of carcinoma bone metastasis. Erythroblastosis is most significant but plasmacytosis is according to the authors the prominent feature. Rohr and Hegglin Nordenson and above all Stöger discussed this relation. Marchal and Mallet found between 3 and 6 per cent of plasmacytes in more than half the cases of carcinoma bone metastasis and often this moderate plasmacytosis was useful to detect micrometastasis lost in the bone marrow and even in some cases permitted discovery of a latent carcinoma of the lung breast stomach or prostate. The morphology of these plasmacytes is indistinguishable from that of the plasma cells in multiple myeloma. The more or less deep basophilia of the cytoplasm the presence or absence of vacuoles or nuclei are the same, multinucleated cells may be found.

When there are only 4 to 6 per cent plasma cells in the bone marrow the histologic differentiation from myeloma is easy. But it is possible to find more than 10 per cent of plasmacytes in metastatic cancer and in 3 cases of prostatic carcinoma between 25 and 50 per cent of the cells were plasma cells. In such cases differentiation from myeloma is very difficult if aggregates of neoplastic cells are not present in the smear. Moreover hyperproteinemia may be present (13.3 grams per cent in one of the cited cases). In such cases the possibility arises that a true myeloma may exist complicating the metastatic carcinoma.

In addition to involvement of the bone marrow a plasmacytic reaction may be found in the liver and was observed by the authors in a case of metastatic carcinoma of the stomach.

J.P.S.

**CIRRHOSE ATROPHIQUE DU FOIE ET MONONUCLÉOSE INFECTIEUSE (ATROPHIC CIRRHOSIS OF THE LIVER AFTER INFECTIOUS MONONUCLEOSIS)** G. Bukel. *Bull. et Mem. Soc. des Hôp. de Paris* 923-927. Séance 8 Oct. 1948.

It is now well known that atrophic cirrhosis of the liver may follow infectious hepatitis and that hepatitis is a common feature of infectious mononucleosis but we had not found any description of atrophic cirrhosis following infectious mononucleosis so this observation seemed interesting to us.

A 38 year old male was affected in February 1946 with typical infectious mononucleosis (with adenopathy enlarged spleen mononucleosis and a Paul and Bunnell reaction positive at a dilution of 1:16).

Recovery was very slow, and in June jaundice appeared which lasted ten days and which reappeared in September. The liver was now enlarged and ascites appeared. Different hepatic tests were strongly pathological. After aspiration of the ascitic fluid the liver was no longer palpable.

After treatment with transfusions plasma methionine vitamin B and Patek diet the patient improved slowly and following seven months of this treatment was in good health.

This patient had never consumed any alcoholic beverage and the authors believe that the succession of mononucleosis and cirrhosis in this case was not a mere coincidence.

J.P.S.

**THE INTERRELATIONSHIP OF HODGKIN'S DISEASE AND OTHER LYMPHATIC TUMORS** R. P. Carter and W. G. Bernhard. From the Army Institute of Pathology Washington D. C. and the Laboratories of the Presbyterian Hospital in Philadelphia. *Am. J. M. Sc.* 216 625-642, 1948.

The authors studied the histology of 1,300 lymphoid tissues submitted to the Army Institute of Pathology during the past war. Of these 700 cases were Hodgkin's disease. They employed the Jackson-Parker classification and distribution was 14.3 per cent paragranuloma 71.1 per cent granuloma and 14.6 per cent sarcoma. The authors presented illustrations of alterations in histologic composition of lesions and discussed their nature and frequency. A virtually complete alteration in histologic pattern of tumors was noted in 39 per cent of 138 autopsied cases in which biopsies were available. In 384 of 700 cases there were a variety of histologic pictures in different areas.

While classification of lymphoid tumors was useful chiefly from the standpoint of prognosis with the increased therapeutic armamentarium it is of particular importance to correlate the histologic picture with therapeutic response. It may be that the variable nature of the lesion is in part responsible for the inconsistencies in response of this group of neoplasms.

C.A.F.

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# BOOK REVIEWS

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*Malra Di Cooley* By G MAOGIONI AND A ASCENZI Rome Abruzzini Editore 1948 Pp 168

The main part of this monograph is devoted to a detailed report of two severe cases of Cooley's anemia. Though the clinical and laboratory features of the disease are carefully studied, the authors were apparently primarily interested in the anatomic-pathologic findings that are reported in great detail.

The literature was searched particularly for cases with special reference to the general anatomic-pathologic findings (21 cases), gross and microscopic findings of surgically removed spleens (15 cases) and anatomic-histo-pathologic findings of the heart (3 cases).

The pathogenetic views currently held in this country are by and large accepted and confirmed by the authors. Excessive hemolysis is regarded as a constant factor, the concept previously accepted in Italy, of the disease as a chronic erythremic myelosis, is discarded in favor of a hemolytic familial disease. Bone changes are regarded as secondary to myeloid.

This work is primarily useful to those interested in the anatomic-pathologic aspects of Cooley's disease.

DAVIDE LIMENTANI

*Hereditry in Human Leukemia and its Relation to Cancer* By AARV IØERBAEK London England H K LEWIS and Co Ltd 1947 Translated from the Danish (Nyt Nordisk Forlag Arnold Busck Copenhagen) Pp 271 plus 8 pp ref

This book is the English translation of Danish research published in 1947. Of the 279 pages, the first 104 are concerned with methods, analysis and discussion. Almost half of the report presents pedigree charts and case histories of the families and individuals involved. A brief summary in Danish is included and a bibliography is appended.

The analysis of the data in this monograph is based on statistical methods which treat the data on a population (i.e. distribution) basis. Such treatment is both descriptive and evaluative and is effectively used on this material.

This study of leukemia in humans was begun in 1945 at the University Institute of Pathologic Anatomy in Copenhagen, Denmark. Two hundred and nine individuals having leukemia were selected from hospital records available in greater Copenhagen and information was gathered on all immediate members of their families as well as uncles, aunts and grandparents. This leukemic group was then matched as closely as possible by a comparable nonleukemic control group of 200 individuals and the corresponding information of their families.

Information gathered by interview was verified by examination of hospital records and death certificates and it was found that the death certificates of 387 relatives of the leukemic probands showed that one of them had died of any of the diseases inquired about. A similar examination of the death certificates of 300 individuals of the control material who were not supposed to have died of cancer showed that four of them had in fact died of that disease. Examination of the supposed cancer diagnoses showed them to be correct in 92 per cent of the cases, while of 687 persons not stated to have died of cancer, 58 per cent were so listed on the death certificates.

There were 17 leukemic probands who had at least one other case of leukemia in their family which could be verified, while the families of the control material had only one case of leukemia. This is a significantly higher incidence of leukemia in the patient material and cannot be attributed to chance. The familial incidence of leukemia in this material is at least 8.1 per cent.

The author believes that the hereditary factors operating in leukemia are common to all the different varieties of the disease because the frequency of the varieties of leukemia observed in 39 families was the same as the incidence of the different varieties of leukemia among 310 nonselected patients.

A significant correlation was found to exist between siblings for age of onset of leukemia. Since it is unlikely that two siblings would show the same disease at the same age by chance, the concept of genetic relationship is strongly supported. The familial incidence which amounts to at least 8 per cent of all cases is more than can be explained by coincidence. The demonstrated relation [of 8 per cent familial incidence] must be supposed to be genetic.

The most likely method of inheritance is believed to be failing dominance but with it due to a single gene or to several (polymaria) is left an open question. On the basis of the present data consisting of thirteen families from this study plus 16 from the literature the author believes that extrachromosomal inheritance may be excluded. Simple dominance and recessive inheritance are also excluded while sex-linked and sex-limited inheritance have not been demonstrated.

The investigation of a possible relationship between pernicious anemia and leukemia showed that in the 209 leukemic proband pedigrees there were 17 verified cases of pernicious anemia i.e. 8 per cent of the families. In the control material pernicious anemia was found in only 6 of the 200 families i.e. 3 per cent. The relationship between leukemia and pernicious anemia may in Videbäck's opinion be due to a hereditary disposition which leukemia and pernicious anemia may have in common with cancer. No genetic relation between leukemia and other diseases of the blood-forming organs was found.

The last section of this paper is devoted to the consideration of the genetic relation between leukemia and cancer. In the data of this study there were 319 cases of cancer (78.9 per cent) among 4041 relatives of leukemic probands while there were 218 cases (5.99 per cent) among 3641 relatives of the control group. The incidence of cancer is about 32 per cent higher in the patient material than in the control group—a statistically significant difference. The conclusion of this section is that a relation does exist between leukemia and cancer evident both in the greater frequency of cancer in relatives of leukemic individuals and also the frequent coexistence of cancer and leukemia in the same patient. Leukemia is therefore believed to be a malignant neoplasm of the blood and the hemopoietic apparatus.

This study is an attempt to answer problems on a factual basis. Though conclusions are few the methods of the study and its objectives are worthy of high praise. Probably few other people recognize as clearly as does the author that much more data from unimpeachable sources is necessary before final conclusions can be reached. Investigators of leukemia and cancer will find occasion to return to this work for it will serve as a useful basis of comparison for their own data.

I. LUDWIN

*Submicroscopic Morphology of Protoplasm and its Derivatives*. By A. FREY WYSSLING. New York: Elsevier Publishing Co. Inc. 1948. Pp. 255, with 38 tables and 160 figs.

This monograph is the second edition of Frey Wyssling's *Submikroskopische Morphologie des Protoplasmas und seiner Derivate* first published in 1938. Extensively revised and rewritten, it has been excellently translated by Prof. J. J. Hermans and Miss M. Hollander.

The clear and exact style of this book makes it a pleasure to read and it should become familiar to all cytologists, cell physiologists, and biophysicists as the best existing presentation of the subject. It will act to the student who is unfamiliar with submicroscopic phenomena as a key to a new world. Even for the specialist, almost every page will be found to contain some piece of unfamiliar and interesting information, but the book is more than a mine of material; it is unusually evocative of ideas for future investigation, many of which will probably come to mind only after it has been read and laid down.

The first section, on the Fundamentals of Submicroscopic Morphology, deals with the organization of solids and the structure of gels; the second section deals with the fine structure of protoplasm (cytoplasm, nucleus, chloroplast, and the erythrocyte) and the last section deals with the fine-structure of the protoplasmic derivatives (cellulose, chitin, fibroin, keratin, collagen, myosin, and starch grains). There is a selected bibliography of over 700 references, together with a subject and an author index. Most of the figures have been introduced to illustrate the spatial arrangements of atoms, molecules, and larger structures discussed in the text; this they do so clearly that it would be possible to become acquainted with the outlines of the subject by studying the figures alone.

ERIC PONDOR

### Erratum

An unfortunate error in the preceding issue of *Blood* (June 1949), in the section on correspondence concerning revised hematologic nomenclature, gives a misleading impression. Page 781, the first sentence following the references to Dr. Osgood's letter should read: "A subsequent letter received from Dr. Jones indicates that Dr. Downey was unable to attend two of the last meetings of the Committee, and that he does not agree on all points with the report." (Instead of "Dr. Downey indicates that he was unable to attend.")

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## *Contributors to this Issue*

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ELIJAH ADAMS, M D

National Institute of Health Senior Research Fellow  
Department of Medicine New Haven Hospital  
New Haven Conn

EDITH BUECHLER, M Sc

Department of Hygiene and Bacteriology Hebrew  
University Jerusalem

J V DACIE, M B , M R C P

Reader in Hematology, Department of Pathology  
Postgraduate Medical School of London London,  
England

CLYMENT A FINCH, M D

Associate in Medicine Harvard Medical School,  
Associate in Medicine Peter Bent Brigham Hospital  
Boston Mass

ALFRED P FISHMAN, M D

Dazian Foundation Research Fellow Michael  
Reese Hospital Chicago Ill

REX G FLUHARTY, Ph D

Research Associate, Physics Department Massachusetts  
Institute of Technology, Cambridge  
Mass

JOHN G GIBSON II, M D

Associate in Medicine Peter Bent Brigham Hospital  
Research Associate in Medicine Harvard  
Medical School Boston Mass

K GUGENGHEIM, M D

Department of Hygiene and Bacteriology Hebrew  
University Jerusalem

EUGENE KAPLAN, M D

Research Associate in Hematology Children's  
Hospital of Michigan Detroit, Mich

J MURRAY KINSMAN, M D

Professor of Medicine, University of Louisville  
School of Medicine, Louisville, Ky

S ROBERT LEWIS, M D

Lieutenant, Medical Corps A. U. S.

S P LUCIA, M D

Professor of Medicine Divisions of Medicine and  
Preventive Medicine, University of California  
Medical School, San Francisco Calif

HAROLD MILLS, M D

Research Fellow in Medicine, Divisions of Medicine  
and Preventive Medicine, University of California  
Medical School, San Francisco Calif

WENDELL C PEACOCK, Ph D

Research Associate, Massachusetts Institute of  
Technology, Cambridge, Mass.

SHEPARD SHAPIRO, M D

Assistant Professor of Clinical Medicine, New  
York University College of Medicine Visiting  
Physician Goldwater Memorial Hospital, New  
York City

MURRAY WEINER, M D

Research Fellow in Medicine, Goldwater Memorial  
Hospital New York City

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# BLOOD

## *The Journal of Hematology*

VOL. IV, NO. 8

AUGUST, 1949

### FAMILIAL HYPOCHROMIC ANEMIA ASSOCIATED WITH POSTSPLENECTOMY ERYTHROCYTIC INCLUSION BODIES

By HAROLD MILLS, M D , AND S P LUCIA, M D

RECENTLY there has occurred a renewed interest in the subject of erythrocytic inclusion bodies, and emphasis has been placed on their appearance following splenectomy.<sup>1,2</sup> At the same time, there has been published an increasing number of reports bearing on the familial and congenital character of various hematologic disorders.<sup>4-7</sup> Therefore, it seems pertinent at this time to report a case of familial hypochromic, microcytic anemia in which large numbers of erythrocytic inclusion bodies were found in the peripheral blood following splenectomy.

#### CASE REPORT

##### *First Entry (7/15-7/17/36)*

B W a 27 year old white married male of German Irish and French descent was first seen at the University of California Hospital on July 15 1936. He had been anemic as long as he could remember but was able to continue normal activity as a grocer and postal clerk until the time of admission. For two months prior to entry he had been plagued by a dull aching left upper quadrant pain associated with moderate weakness and dyspnea. His physician found the hemoglobin to be 26 per cent ordered transfusions and then referred him to the University of California Hospital for diagnosis.

*Family History* His family history revealed that his mother died of Bright's disease and his maternal half brother (then 17 years of age\*) had also suffered from anemia. An examination of the patient's

From the Divisions of Medicine and Preventive Medicine University of California Medical School  
San Francisco Calif

\*Through the courtesy of Mr Harry Potter Registrar of the Veterans Administration Hospital at Wood Wisconsin we have learned that the maternal half brother of our subject suffered from hypochromic anemia refractory to treatment. He was splenectomized on June 6 1945 and expired on September 17 1947 of anemia, with widespread thrombophlebitis peripheral emboli and cardiac failure. Despite many transfusions the erythrocyte count and the hemoglobin content fluctuated around the average of 3 000 000 and 8.5 grams respectively pre and postsplenectomy. The white counts varied between 15 000 and 25 000 with a differential count which averaged 25 per cent PMN's and 64 per cent lymphocytes. There was a moderate degree of anisocytosis poikilocytosis and hypochromasia of the erythrocytes. The platelet count was 24 000 per cu mm. The erythrocyte fragility test revealed hemolysis from 0.46 to 0.32. The Wintrobe indices were as follows: Volume index 1.0 color index 0.85 MCHc 25 per cent MCV 104. A Price Jones curve of the erythrocytes (done by H.M.) revealed a mean corpuscular diameter of 7.62. Unfortunately no correlative blood counts were available. MCHc conc 0.25 saturation index 0.84. The sedimentation rate was 6 mm/hour. The reticulocyte count was 0.2 per cent. The urinary



infant son (age 17 months) revealed a mild anemia with moderate anisocytosis. The blood count was Hemoglobin 74 per cent R B C 3,640,000 W B C 11,800—PMN Fil 11 per cent, PMN Nonfil 35 per cent, eosinophiles 1 per cent lymphocytes 79 per cent monocytes 5 per cent and myelocytes 1 per cent.

*Past History and System Review.* The past history and system review were noncontributory except for measles, chickenpox, whooping cough and smallpox acquired in childhood.

*Physical Examination.* The physical examination was essentially noncontributory except for slight cardiomegaly associated with a loud systolic apical, hemic murmur, a questionably palpable liver and a firm sharp-edged spleen felt 3 fingerbreadths below the lateral costal margin.

*Laboratory Data.* Hemoglobin 45 per cent R B C 2,790,000 W B C 5,120 (with a normal differential count). The erythrocytes revealed anisocytosis, poikilocytosis and achromia and the platelets were said to be increased. Observations on the urine, gastric content and erythrocyte fragility (Hamburger method) were normal. The Rose Bengal test (biliary excretion) and phenylsulphathiazole test were within normal limits. Roentgen examination of the gastrointestinal tract and chest revealed no abnormalities.

A diagnosis of chronic hypochromic microcytic anemia associated with splenomegaly (2 first stage Banti's syndrome) was made and splenectomy was recommended.

#### *Second Entry (9/22/36-2/19/37)*

*Interval History.* The patient was splenectomized by his local physician and returned to work two weeks later. He was asymptomatic until two weeks before the second entry at which time he complained of exertional dyspnea and noticed swelling and pain in his left thigh. Within the next week, the pain and swelling involved the left leg and the right thigh. Five days prior to the second hospital admission he suffered an attack of acute pleuritic pain in the right anterior chest. The pain subsided gradually, and there was no hemoptysis.

*Physical Examination.* The findings on physical examination were essentially the same as previously noted except for the presence of a well healed splenectomy scar and the signs of thrombophlebitis in both thighs and calves.

*Laboratory Data.* The hemoglobin varied from 12 to 60 per cent, the R B C from 900,000 to 3,000,000 and the W B C from 12,000 to 40,000 with a terminal drop to 7,500. The morphology of the leukocytes was always within normal limits and their differential counts revealed a slight increase in the percentage of polymorphonuclear cells. The platelet counts showed variations of 900,000 to 1,900,000. A Price-Jones curve of the erythrocytes (fig. 1) gave the following results: Mean erythrocyte diameter  $6.65 \mu\text{m} \pm 1.5$  (50 per cent were below  $7.0 \mu\text{m}$  in size and 35.6 per cent were  $6.0 \mu\text{m}$  or smaller). The erythrocytes revealed anisocytosis, poikilocytosis and hypochromasia. Twenty-four to 67 per cent of the erythrocytes contained inclusion bodies (fig. 2) which gave a positive prussian blue reaction. (This was demonstrated by treating blood films with a mixture of equal parts of 2 per cent potassium ferrocyanide and 2 per cent hydrochloric acid for twenty minutes, then washing and counterstaining with safranin.) Frequent observations on the bleeding time and the erythrocyte fragility failed to reveal any abnormalities. On occasion the coagulation time (Lee and White) was slightly hastened. A clot retraction test showed the clot to be markedly retractile (1 hour and 35 minutes for complete retraction). The urine was negative for urobilinogen, urobilin and bile on numerous occasions.

*Course in Hospital.* Despite intensive supportive therapy, including numerous transfusions, large doses of ferrous sulfate and large doses of liver extract (refined and crude) the patient expired on February 19, 1937. During his final hospitalization the following complications were noted:

1. On November 1 and on November 9, 1936, the subject presented symptoms suggestive of pulmonary infarct.

urobilinogen was 0.1 mg. per 100 cc. The icteric index was 8 and the serum bilirubin 0.16 mg. Examination of his peripheral blood smear and bone marrow (fig. 3) revealed erythrocytic inclusions in large numbers of the erythrocytes and normoblasts. These inclusions gave a positive reaction for iron. The clinical diagnosis was Anemia, primary idiopathic. At death the autopsy revealed the following pertinent findings: (1) Anemia profound primary type. (2) Posterior myocardial infarct recent with mural thrombosis and secondary cerebral and peripheral arterial emboli. (3) Hemosiderosis of the liver, spleen, pancreas and abdominal lymph nodes. (4) Fibrous thrombophlebitis of the left femoral vein.

— On November 25 he experienced pain in the right tonsillar region. Examination revealed blanching of the superior  $\frac{2}{3}$  of the right tonsil and of both the anterior and posterior faucial pillars. One week later the left tonsillar region became similarly involved. Thrombosis of the arterial supply to the tonsillar

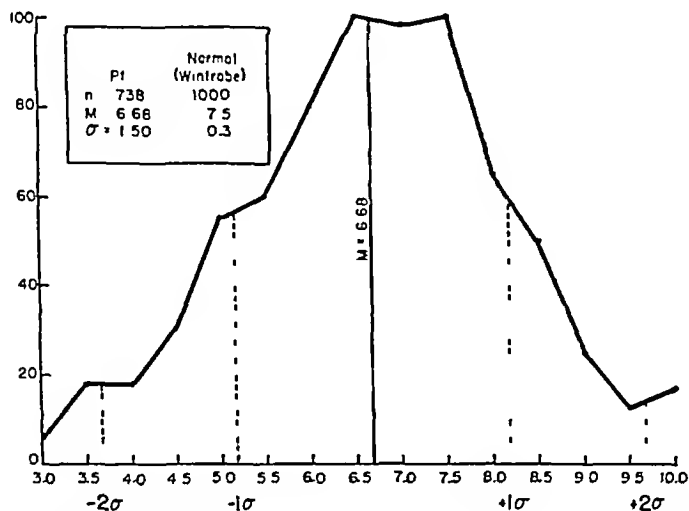


FIG. 1.—PRICE JONES CURVE (PATIENT B W) Vertical axis indicates number of RBC horizontal axis indicates diameter of RBC

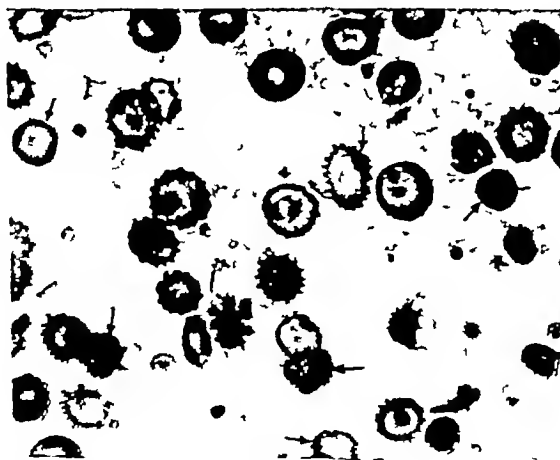


FIG. 2.—MICROSCOPIC VIEW OF SIDEROCYTES IN PERIPHERAL BLOOD OF CASE PRESENTED (Prussian blue stain counterstained with safranin  $\times 650$ ) Note small coccoid siderotic granules within the siderocytes

region was suspected in both instances and this suspicion was substantiated when within two weeks the upper  $\frac{2}{3}$  of both tonsils became sharply demarcated and sloughed out without hemorrhage or ulceration

- 3 On December 26, thrombosis of the left ante-cubital vein was noted
- 4 On January 12 1937 he experienced a bout of paroxysmal auricular fibrillation which lasted three hours

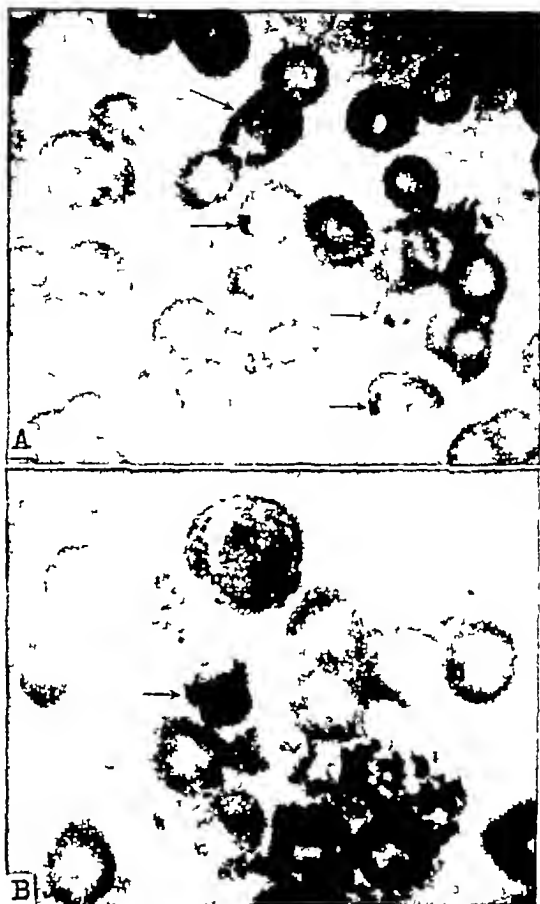


FIG 3 —(A) Microscopic view Wright's stain ( $\times 650$ , enlarged approximately  $\times 2$ ) revealing siderocytes in the peripheral blood of our subject's maternal half brother (B) Microscopic view of bone marrow of our subject's maternal half brother. Note siderotic material within normoblast

- 5 On January 15 edema of the left calf was noted which subsequently involved the entire left lower extremity and the adjacent abdominal wall to the level of the costal margin. This was considered to be due to thrombosis of the left common iliac vein.

6 Before death the anemia became more marked and generalized anasarca appeared.

*Autopsy Findings* A summary of the important features of the autopsy, which was performed by Dr C. L. Conner, follows:

## Anatomic Diagnosis

A Hemolytic anemia with (1) Splenomegaly (study of surgical specimen), (2) hyperplastic bone marrow (3) lymph nodes showing minor erythropoiesis (4) hemochromatosis of liver, pancreas, lymph node and bone marrow (5) generalized edema (6) terminal heart failure

B Chronic proliferative endarteritis pulmonary and other arteries

C. Pulmonary and left iliac thrombosis

D Infarcts of lungs and spleen (pre-operative specimen)

Antemortem thromboses were found in the right auricle the distal end of the incised splenic vein and in the left iliac vein with an extension into the inferior vena cava. No thrombi were found in the mesenteric vessels but two pulmonary infarcts were present.

A special note draws particular attention to the following. All tissues of the body contain a great deal more liquid than normal. While no definite edema is noted externally in the thigh for instance when the tissues here are cut through and manipulated a great deal of liquid can be expressed from them. This is true of other apparently non-edematous tissue as well. The whole body appears to be water logged. The blood is quite liquid and watery and shows an obvious extreme anemia.

The microscopic examination of the hematopoietic system is as follows:

*Spleen* Sections of the spleen show a diffuse increase in pulp so that the splenic follicles are widely separated from one another although the latter are not diminished in size. The pulp is made up of erythrocytes of all shapes showing remarkable poikilocytosis and all degrees of degeneration and hemolysis. There are at the same time many normoblasts present and a scattering of myeloid cells. There is no increase in fibrous tissue within the spleen but the capsule is somewhat thickened. An infarct with beginning organization around the edges is present. (Note: The spleen weighed 790 grams and two small accessory spleens were present. The organ was dark red in color.)

*Lymph Node* Several lymph nodes show edema without much other change. Others contain a large amount of pigment in phagocytic cells within the sinusoids. There is some increase in immature cells among the lymphocyte follicles outside of germinal centers. Some of these having small black nuclei may be young erythroblasts.

*Bone Marrow* This is definitely hyperplastic with most of the young cells appearing to belong to the erythroblastic series. Other immature cells are present in small numbers and there is the usual number of megakaryocytes. There is also a great deal of pigment [chiefly hemosiderin] scattered throughout.

*In summary* The immediate cause of death is obviously the profound edema of the lungs and heart failure with edema of all tissues although the remarkable amount of liquid in the interstitial tissue cannot be due to heart failure alone. This is undoubtedly due to an anoxia associated with profound anemia and an increase in the hydrophilic nature of muscle and connective tissue proteins. The underlying physiological change has apparently been an increase in hemolysis of red blood cells even though these are not more fragile than normal. The result has been anemia with erythropoietic activity of the spleen and bone marrow and to a less extent lymph nodes. Another effect has been the deposition of blood pigment in liver, pancreas, lymph node and bone marrow leading to a type of hemochromatosis. The ultimate etiological factors in all this are not apparent. There is also no explanation for the intimal proliferation and thromboses of vessels noted.

Aided by Dr. Stuart Lindsay (Department of Pathology), the sections were reviewed using the prussian blue and Giemsa stains. Since large amounts of hemosiderin in the cells and interstitial tissue of the reticulo-endothelial system gave the positive prussian blue reaction, it was impossible to identify any granules which might represent phagocytosed erythrocytic inclusions. A moderate amount of non-staining brown pigment was also present. Using the classification of Rath and Finch,<sup>3</sup> an abnormal amount (Grade IV) of hemosiderin was found in the sternal bone marrow.

The clinical and autopsy findings recorded above may be interpreted as follows:

The results of the postmortem examination are consistent with the diagnosis of hemolytic anemia, but this diagnosis is not compatible with the clinical and laboratory data. The data fit best in the diagnostic category of *Hypochromic, microcytic anemia secondary to a congenital defect in the ability of the hemopoietic system to utilize iron*. To support this diagnosis the following points are worthy of emphasis: (1) The absence of clinical or laboratory evidence of hemolysis. (2) An anemia of long duration, refractory to the usual therapeutic agents. (3) The family history of a maternal half-brother who suffered from a similar type of lethal anemia. (4) The absence of reticulocytosis despite large doses of iron as well as the presence of a surfeit of iron in the tissues.

The widespread hemosiderosis may have been due in part to multiple transfusions. Rath and Finch<sup>8</sup> have demonstrated excessive amounts of hemosiderin in the bone marrows of patients who had received multiple transfusions. A more reasonable explanation of the hemosiderosis in this case is that its deposition in the tissues is an expression of the inability to utilize iron despite its presence in adequate amounts. Wintrobe et al.<sup>9</sup> were able to produce, in swine fed a diet deficient in pyridoxine, a microcytic anemia which was associated with hyperferremia, a normal icteric index and hemosiderosis of the spleen, liver, and bone marrow. They believed that the anemia was due to faulty utilization of iron. A similar mechanism may operate to produce anemia in the human subject.

## DISCUSSION

### *I Definition of Inclusion Bodies*

In the broad sense, erythrocytic inclusion bodies may be defined as intracorpuscular structures having certain morphologic and tinctorial characteristics. The definition may be limited further by the recognition of two categories, false and true inclusions. The former are predominantly artefacts produced in the laboratory. The true inclusion bodies are structures which may be either nuclear or cytoplasmic remnants, the products of normal metabolic processes, or the result of some aberration in the metabolism of the various constituents within the erythrocytes. Accordingly inclusion bodies may be classified as

#### **I False Inclusion Bodies of the Erythrocyte**

##### **A Those produced by faulty technique**

1. Unclean equipment
2. Unfiltered stain

##### **B Those produced by subjecting blood films to certain chemical or physical agents<sup>10, 11</sup>**

##### **C Cabot's rings (?)<sup>12</sup>**

##### **D Bacteria and parasites**

#### **II True Inclusion Bodies of the Erythrocyte**

##### **A. Nuclear or cytoplasmic remnants<sup>13</sup>**

1. Howell Jolly bodies
2. Diffuse basophilia
3. Reticulocytic material

##### **B Those containing iron (siderocytes)<sup>14</sup>**

1. An expression of faulty iron metabolism<sup>1, 2, 3</sup>

- a Due to toxins
  - i Acquired hemolytic anemia<sup>3 13</sup>
  - ii Stippling found in lead poisoning<sup>3 13</sup>
  - iii Associated with bacterial toxemias severe burns or industrial solvent poisoning<sup>13</sup>
- b Due probably to a congenital defect in iron metabolism
  - i Familial hypochromic microcytic anemia<sup>4</sup>
  - ii Familial hemolytic icterus<sup>3 16</sup>
  - iii Anemia associated with flexed tail and belly spot in mice<sup>14</sup>
- 2. An expression of normal iron catabolism
  - a Siderocytes found in aging blood<sup>17</sup>
- 3 Unclassified
  - a. Those found in association with Banti's syndrome and other hematologic disorders<sup>1 15</sup>
- C Those found in toxic or deficiency conditions
  - i Heinz bodies of toxic irreversible anemias<sup>18 19 20</sup>
  - 2. Inclusion bodies of atabrine poisoning<sup>21</sup>
  - 3 Inclusion bodies found in pyridoxine deficiency<sup>2</sup>—may be siderocytes<sup>2</sup>

### *False Inclusion Bodies*

False inclusion bodies are artefacts of the erythrocyte, animate or inanimate, which may at times assume certain definitive patterns. Rinehart<sup>10 11</sup> treated blood films with a mixture of reduced phosphomolybdic acid and either potassium dichromate or phosphotungstic acid and was able to produce various well defined patterns of hemoglobin precipitation. More recently, Schleicher<sup>12</sup> has demonstrated that the Cabot's ring bodies are probably artefacts produced in the laboratory. He concluded that they represented denatured protein configurations produced by subjecting the red blood corpuscle to hemolytic agents.

We have examined preparations of bone marrow and peripheral blood in numerous hematologic conditions and have encountered occasional single inclusion bodies. Their significance remains in doubt, because they may be confused with spurious inclusion bodies produced by amorphous precipitate (a common occurrence with unclean cover-slips) or the precipitate from unfiltered stain. Frequently, artefacts are encountered which are semirefractile and which assume a bluish-purple appearance upon change of focus. Therefore, it is stressed that predictable morphologic, chemical and tinctorial characteristics must be satisfied before an erythrocytic inclusion may be classified within the category of true inclusion bodies.

In some circumstances, microorganisms such as streptococci and staphylococci may become attached to the surface of the erythrocyte and give the appearance of an inclusion body. On the other hand, other microorganisms, as the Bartonella and the Plasmodia of malaria, specifically enter the erythrocyte to produce inclusion bodies. Finally, it is possible that the products of metabolic change induced by microorganisms or viruses may simulate inclusion bodies. All such instances may be classified as examples of false inclusion bodies.

### *True Inclusion Bodies*

Enumerated among the true inclusion bodies of the erythrocyte are the Howell-Jolly body (probably a nuclear remnant), the granules of diffuse basophilia, and the reticulocytic material (probably the basophilic remains of spongioplasm)<sup>12</sup>

The nature of basophilic stippling in lead poisoning is still uncertain, although studies by Case<sup>18</sup> and MacFadzean and Davis<sup>3</sup> indicate that the stippling is caused by iron-containing granules, thus making the stippled cell basically similar to the siderocyte.

True inclusion bodies not containing iron are observed in the anemias secondary to such toxic agents as atabrin, erythrol-tetranitrate and sulfonamides. Mushett and Siegal<sup>21</sup> produced anemia and erythrocytic inclusions in rats, mice and hamsters by feeding them large doses of atabrin. These inclusions stain blue with Wright's stain, and give a negative reaction for iron.<sup>3</sup> They were also noted within the lymphocytes—a phenomenon not observed in studies of iron-containing inclusions.

The Heinz-body characteristic of irreversible toxic anemia has been described in the German literature. More recently, Fertman and Doan<sup>18, 19</sup> have reported the case of an elderly man who had been taking erythrol tetranitrate and in whom there appeared a fatal refractory anemia characterized by erythrocytes containing Heinz bodies. Figge<sup>20</sup> was able to reproduce Heinz bodies in 90 per cent of the erythrocytes of mice by feeding them a 0.3 per cent solution of sulfanilamide in distilled water. He concluded that the tendency of various sulfonamides to induce Heinz bodies paralleled their ability to produce hemolytic anemia. The Heinz body is best seen in supravitral preparations, does not stain with Wright's stain and gives a negative prussian blue reaction.

Wintrobe et al.<sup>9</sup> fed pigs a diet deficient in pyridoxine following which the animals developed a microcytic anemia. The anemia was associated with a rise in serum iron concentration and hemosiderosis of the spleen, liver and bone marrow. There was no associated rise in the icteric index. As the anemia developed, the erythrocytes were found to harbor a moderately large blue-staining granule resembling a nuclear particle.

## II *The Problem of Iron Containing Inclusion Bodies*

The most important of the true inclusion bodies of the erythrocyte are those which give a positive prussian blue reaction indicative of the presence of iron. These cells were called siderocytes by Grüneberg,<sup>14</sup> who first described them in observations made on the anemia associated with the flexed-tail and belly-spot phenomenon of mice. He found that the erythrocytes of these animals at birth contained large numbers of inclusion bodies. As the animals matured, the anemia characteristic of the condition subsided, and there was a concomitant decrease in the number of siderocytes found in the peripheral circulation. Grüneberg also demonstrated that the fetuses of normal mice harbor siderocytes which disappear shortly after birth, and he also observed siderocytes in the heart blood of a human fetus (14 weeks old), as well as in the blood of four premature and full term human fetuses (33 to 40 weeks old).<sup>22</sup>

Pappenheimer, Thompson, Parker and Smith<sup>2</sup> reported 3 cases of anemia with splenomegaly, 2 of acquired hemolytic anemia and 1 an undetermined type of anemia. After splenectomy, examination of the peripheral blood of these subjects revealed large numbers of erythrocytes containing inclusion bodies. In an attempt to define the nature of these, Pappenheimer et al. showed that the bodies gave a

positive prussian blue reaction when stained in blood smears as well as in sedimented laked blood. The bodies were anisotropic, gave a negative Feulgen reaction (for nucleic acid) and the iron they contained was neither ferritin nor hemosiderin. The inclusions were gram-negative, did not stain with hematoxylin, did not contain alkaline phosphatase, nor did they fix complement. When a sample of heparinized blood was placed in a magnetic field, erythrocytes containing the inclusion bodies became concentrated along the line of magnetic contact. A significant number of granules morphologically similar to those observed within the erythrocytes were noted in the reticulo-endothelial cells (Kupffer cells, histiocytes and splenic endothelial cells) of two subjects and in the third, they were encountered in small numbers within the splenic endothelial cells.

MacFadzean and Davis,<sup>2</sup> stimulated by the work of Pappenheimer,<sup>2</sup> have published a comprehensive study and review of erythrocyte inclusion bodies, and emphasize their importance in acquired hemolytic anemia. They state that the inclusions are most commonly coccoid granules varying in size from 0.5 to 2.0 micra in diameter and usually located in the periphery of the corpuscle. Their form is frequently bacillary or tadpole-shaped and occasionally they assume amoeboid, diploid and tetrad forms. Should more than one body be found within the corpuscle (especially common following splenectomy), variation in size is the rule. The granules may form a solid mass of material and leave only a thin rim of hemoglobin in the periphery of the erythrocyte. Furthermore, they state that erythrocytes which contain large numbers of inclusion bodies tend to be smaller than normal. When viewed in unstained preparations, the inclusions appear as refractile colorless structures, when stained with the Leishman, Wright, or Giemsa preparations, they stain a purplish-blue although light blue and reddish-purple forms are sometimes observed as well as rodlike forms which stain alternately light and dark. The most important characteristic of the inclusion body they describe is its positive reaction for iron.

MacFadzean and Davis examined the bone marrow of patients whose peripheral blood contained erythrocytic inclusion bodies and noted their presence only in those cells of the erythroid series which were hemoglobinized. Cells showing minimal hemoglobinization were said to contain inclusions that were in close proximity to the nucleus, but as hemoglobinization increased, the granules gradually shifted toward the periphery, the position which they occupy in mature erythrocytes. Occasionally granules identical with those described within the red blood corpuscles were seen lying free in the marrow or within monocytes and reticulum cells, but not within any of the other varieties of leukocytes. They also noted granules within the endothelial cells of the spleen.

MacFadzean and Davis described 7 cases of acquired hemolytic anemia, 6 of which had been splenectomized. Before splenectomy, the maximum siderocyte count was 11 per cent or less, and following splenectomy it varied from 16 to 88 per cent. These authors postulate that corpuscles containing inclusions are defective cells rapidly eliminated from the circulation by the reticulo-endothelial system, especially that of the spleen. In support of this hypothesis they present some interesting data. Before splenectomy, they found inclusion bodies within a



large number of bone marrow normoblasts, and only small numbers of inclusion bodies within the erythrocytes of the peripheral blood. In 4 patients who were examined after splenectomy, the number of affected corpuscles in the peripheral blood was found to be increased and more closely approached the number of affected normoblasts in the bone marrow. They state, "it is evident that the rise in the total red cell count, for a variable period after splenectomy, can be accounted for entirely by the increase in the absolute number of inclusion-containing erythrocytes, since the number of unaffected cells showed little change."

Case<sup>17</sup> has investigated the occurrence of siderocytes in blood of cats, dogs, and human beings, stored outside the body. He found that siderocytes appeared with regularity as the stored blood aged, although certain agents modified the rapidity of their appearance. Depressed temperatures, carbon monoxide and glucose inhibited the rate of siderocyte formation, whereas heat and hemolytic agents such as phenylhydrazine accelerated the process. As siderocytes appeared, granules were seen to lie free in the plasma, and leukocytes, especially macrocytes, phagocytosed the siderotic material. A normal human volunteer was given phenylhydrazine following which the red blood cell count and hemoglobin content of the blood became decreased, and the blood and urinary siderocyte count rose. With recovery and the associated appearance of young cells, the siderocytes disappeared. Case concluded from these experiments that all erythrocytes go through a siderocyte stage during which they are susceptible to phagocytosis. Furthermore, he states that siderocytes are probably old cells and that the siderotic material is catabolic iron. He also is of the opinion that the siderotic granules he observed in the erythrocytes were probably different from the granules described by Pappenheimer.<sup>2</sup>

In an examination of 279 blood samples from normal persons, Case<sup>18</sup> found a maximum siderocyte count of 0.8 per cent while MacFadzean and Davis<sup>3</sup> failed to find any inclusions in 62 peripheral blood smears taken from normal individuals. Case also demonstrated siderocytes in hypochromic microcytic anemia, hemochromatosis, bacterial toxemias, severe burns, industrial solvent poisoning, lead poisoning, untreated pernicious anemia, sickle cell anemia and acholuric jaundice. The author states that all of these conditions, except hemochromatosis, are hemolytic processes and that the siderocyte levels follow the severity of hemolysis rather closely.

The papers of MacFadzean and Davis<sup>3</sup> and Pappenheimer et al.<sup>2</sup> stress the importance of splenectomy as the factor which precipitates the appearance of erythrocytes containing inclusion bodies, and the former emphasize the fact that the greatest number of these cells are found in subjects suffering from acquired hemolytic anemia who are splenectomized. Doniach, Grunberg and Pearson<sup>1</sup> have reported a case of Banti's syndrome in which the peripheral blood contained 30 per cent siderocytes following splenectomy. These investigators found 1 per cent or less siderocytes in the peripheral blood after splenectomy in a case of thrombocytopenic purpura, two cases of traumatic rupture of the spleen, and one case of possible splenic anaemia. Pappenheimer<sup>2</sup> demonstrated granules similar to erythrocyte inclusion bodies in the splenic sinus endothelium of a majority of cases of thrombo-

cytopenic purpura and rheumatic fever, and to a lesser extent in Banti's syndrome and hemolytic jaundice.

Otto and Rezek<sup>23</sup> report a case of lethal anemia associated with fever, splenomegaly, leukopenia and thrombocytopenia. A moderate number of erythrocytic inclusion bodies were observed preoperatively in the peripheral blood, and they became markedly increased in number following splenectomy. Although these inclusions fitted the description of siderotic granules, they believed them to be Bartonella bodies, despite the negative results of extensive cultural and inoculation studies. Horne, Lederer, Kirkpatrick, and Leys<sup>16</sup> report a family (diagnosis: congenital hemolytic disease) in which 6 members developed hemolytic crises within a few days. During the crises, many of the erythrocytes were observed to contain inclusions which they believed were Howell-Jolly bodies. The inclusion bodies they describe satisfy the morphologic characteristics for siderocytic inclusions. Unfortunately, the inclusions were not stained for iron by either Otto and Rezek or Horne et al.

Examination of the peripheral blood of our subject revealed that 24 to 67 per cent of the erythrocytes contained inclusion bodies (fig. 2). The inclusions stained purplish-blue with Wright's stain, and after decolorization and restaining gave a positive prussian blue reaction. Morphologically, they were similar to those described by the authors mentioned above. Although occasional cells were seen in which one granule only was visible, the majority contained multiple granules, and some cells contained five or more inclusions which formed a solid mass of material within the cell. Most of the inclusions were coccoid, although other forms were occasionally seen. Examination of the bone marrow and peripheral blood of a maternal half-brother (see footnote, pp. 891-2) revealed inclusion bodies in both the normoblasts and erythrocytes, the inclusions being similar in morphology to those observed in the erythrocytes of our subject (fig. 3). Unfortunately, specimens of bone marrow before splenectomy were not available for examination.

In summary, the inclusion bodies demonstrated in the erythrocytes of our subject (and his maternal half-brother) and those described in the papers of Grüneberg,<sup>1</sup> Case,<sup>18</sup> Pappenheimer et al.,<sup>2</sup> and MacFadzean and Davis,<sup>3</sup> all gave a positive iron reaction. This characteristic places them in the category of siderocytes. It seems likely that the siderocytes described by Case<sup>17</sup> and found in aging blood do not have the same fundamental significance as the inclusion bodies described by other workers. The relationship between the siderocytes which Grüneberg<sup>14</sup> found in fetal human blood and in the anemia of mice exhibiting the flexed-tail and belly-spot phenomenon and those found in human disease processes will have to await further clarification.

### III The Significance of Heredity in Anemia

In regard to the familial characteristic of the anemia observed in this instance, it would be advisable to emphasize the distinction between congenital and hereditary anemias. A congenital anemia may be defined as one existing at birth and acquired *in utero*. The anemias of this type may be due to metabolic disturbances, nutritional

defects, or iso-immunization phenomena. An hereditary anemia may be defined as one due to a constitutional defect, transmitted from parent to offspring. Familial hemolytic icterus, sickle cell anemia and Mediterranean anemia are examples of hereditary anemias. The mechanism of their transmission has been recently clarified.

Valentine and Neel<sup>6</sup> have demonstrated that Mediterranean anemia exists in two forms, one, thalassemia minor, a relatively benign disease characterized by mild anemia, ovalocytosis, target cells, the frequent occurrence of mild splenomegaly and a good prognosis, the other, thalassemia major, is characterized by more prominent features and a poor prognosis. These authors offer the hypothesis that thalassemia minor is due to heterozygosity for a factor which when homozygous results in thalassemia major.

The problem of ovalocytosis (elliptical erythrocytes) has been studied by Wyandt, Bancroft and Winship<sup>7</sup> and shown to be an hereditary trait more frequent in males than in females. Although they consider the anomaly to be a benign manifestation, a more recent review<sup>24</sup> emphasizes the fact that occasionally ovalocytosis may be associated with anemia.

Haden<sup>5</sup> has reported two families in which he found 8 patients afflicted with congenital hemolytic anemia without spherocytosis. The erythrocytes tended to be macrocytic and the fragility tests were within normal limits. A prominent feature in one family was the high percentage of stippled cells. Rundles and Falls<sup>4</sup> have reported two families in which the male members, through several generations, showed hypochromic, microcytic anemia associated with deformed erythrocytes. The females of these families appeared to transmit the disease. None of them suffered from anemia, although many had splenomegaly and minor red cell deformities such as ovalocytosis. A male subject of one of the families was splenectomized because of severe anemia, and following the removal of the spleen, inclusion bodies similar to those described by Pappenheimer were noted within the erythrocytes. They also report the case of a male unrelated to the two families, who was splenectomized, following which 40 per cent of the corpuscles were noted to contain inclusion bodies. The subject died one year later and the autopsy revealed diffuse hemochromatosis. Rundles and Falls consider this type of anemia to be a sex linked abnormality, the female carrying the recessive or incompletely recessive gene.

In view of these data, it is probable that the subject of this report falls within the category of hereditary anemia described by Rundles and Falls. The history of anemia since childhood and the similar hematologic and clinical course of his maternal half-brother are offered as evidence for the assumption. The mild anemia found in the infant son of our subject is somewhat out of line with the hypothesis since his mother was hematologically normal. However, this information should not materially affect the above conclusion.

#### *IV The Relation of Postoperative Thrombocytosis to Thrombosis*

A third most interesting feature of this case was the marked and persistent post operative thrombocytosis (as high as 1,900,000), associated with clinical and pathologic evidence of thrombophlebitis. Rosenthal<sup>25</sup> and Evans<sup>6</sup> were among the

first to show that splenectomy is frequently followed by postoperative thrombocytosis. Furthermore, Dawbarn, Earlam and Evans<sup>7</sup> have shown that any major operation, including childbirth and especially caesarean section, may be followed by a rise in the platelet count which reaches a maximum about the tenth post-operative day and subsequently declines toward normal. Adams<sup>8</sup> confirmed the work of Dawbarn et al. in regard to postoperative thrombocytophilia, and reported that 4 of 5 patients who exhibited postoperative platelet counts above 1,000,000 did not have clinical evidence of thrombosis. The relationship between thrombocytosis and siderocytosis is not within the domain of this paper, although they may bear a common relationship to the problem of thrombosis, a prominent feature of the case given in this report as well as that of his maternal half brother.

### SUMMARY

A case is presented of familial, hypochromic, microcytic anemia, associated with the appearance of siderocytes in the peripheral blood following splenectomy. The medical literature of the recent past focuses attention on the clinical recognition of inclusion bodies, but their origin and significance have not been completely clarified. Rundles and Falls<sup>4</sup> were the first to demonstrate them in hereditary hypochromic microcytic anemia, and in addition they have been shown to appear in acquired hemolytic anemia,<sup>2</sup> Banti's syndrome,<sup>1</sup> lead poisoning,<sup>3</sup> and in hemochromatosis, bacterial toxemias, industrial solvent poisoning, sickle cell anemia and acholuric jaundice.<sup>16</sup>

It is probable that the anemia in the case under discussion may have been due to some defect in iron metabolism. Neither the subject nor his maternal half-brother were demonstrated to have any objective evidence of hemolysis, and neither revealed reticulocytosis, despite intensive iron and liver therapy, a point in favor of poor utilization of iron. Furthermore, marked hemosiderosis was an outstanding feature of both cases signifying that at least one form of storage iron was available but not utilized.

The significance of inclusion bodies within erythrocytes is discussed and a classification of inclusion bodies is offered. A final statement regarding the nature of iron granules within red cells must await further research. When present within the erythrocytes of the peripheral blood or in the erythroid series of the bone marrow, they probably are the result of faulty iron metabolism either due to some inherent defect or secondary to the action of some noxious agent. Their prognostic significance is obscure, but this is probably related to the severity of the disease process of which they are an expression.

### REFERENCES

- <sup>1</sup> DONIACH I, GRÜNEBERG H AND PEARSON J E. J. The occurrence of siderocytes in adult human blood. *J. Path. & Bact.* 55: 23, 1943.
- <sup>2</sup> PAPPENHEIMER A M, THOMPSON W P, PARKER D D AND SMITH K. E. Anemia associated with unidentified erythrocytic inclusions after splenectomy. *Quart. J. Med.* 14: 75, 1945.
- <sup>3</sup> MACFADZEAN A J S AND DAVIS L J. Iron staining erythrocytic inclusions with special reference to acquired haemolytic anaemia. *Glasgow M. J.* 28: 237, 1947.
- <sup>4</sup> RUNDLES R. W. AND FALLS J. F. Hereditary (sex linked) anemia. *Am. J. M. Sc.* 217: 641, 1946.

- <sup>5</sup> HADEN, R. L. A new type of hereditary hemolytic jaundice without spherocytosis *Am J M. Sc.* 214 255 1947
- <sup>6</sup> VALENTINE W. N. AND NEEL, J. V. Hematologic and genetic study of the transmission of thalassemia *Arch Int Med* 74: 185, 1944
- <sup>7</sup> WYANDT H. BANCROFT P. M. AND WINTSHIP T. O. Elliptic erythrocytes in man *Arch Int. Med.* 68 1043 1941
- <sup>8</sup> RATH C. E., AND FINCH, C. A. Sternal marrow hemosiderin *J Lab & Clin Med* 33 81 1948
- <sup>9</sup> WINTROBE M. M. FOLLIS, R. H., MILLER M. H. STEIN H. J. ALCAYAGA R. HUMPHREYS, S. SUKTA, A. AND CARTWRIGHT G. E. Pyridoxine deficiency in swine *Johns Hopkins Hosp Bull* 72 1, 1943
- <sup>10</sup> RINEHART J. F. Intracellular precipitation and impregnation of hemoglobin in the erythrocyte. *Folia Hemat* 48 231 1932
- <sup>11</sup> ——— Unusual structures in the erythrocyte *Anat Rec* 52 151 1932
- <sup>12</sup> SCHLEICHER, E. M. Origin and nature of Cabot ring bodies of erythrocytes *J Lab & Clin Med.* 27 983 1942
- <sup>13</sup> WINTROBE M. M. *Clinical Hematology* ed 2. Philadelphia Lea & Febiger p 58 ff
- <sup>14</sup> GRÜNEBERG H. Siderocytes a new kind of erythrocyte *Nature* 148 114 1941
- <sup>15</sup> CASE R. A. M. Siderocytes in haematologic diseases *J Path & Bact* 57 271, 1945
- <sup>16</sup> HORNE J. L., LEDERER H. KIRKPATRICK H. J. R., LEYS D. G. Familial crises in congenital haemolytic disease *Lancet* 2 33 1945
- <sup>17</sup> CASE R. A. M. Siderocytes in mammalian blood *Nature* 152 599 1943 and *Proc Roy Soc., s. B* 133 235 1946
- <sup>18</sup> FERTMAN M. H. AND DOAN C. A. Irreversible toxic inclusion body anemia A clinical and experimental study *Proc Central Soc Clin Research* 18 66 1945
- <sup>19</sup> ——— AND ——— Irreversible toxic inclusion body anemia a rarely recognized syndrome, *Clinical and experimental studies* *Blood* 3 349 1948
- <sup>20</sup> FIGGE F. H. J. Studies on the nature and significance of Heinz bodies in erythrocytes *Anat Rec.* 94 461 1946
- <sup>21</sup> MUSHETT C. W. AND SIEGEL, H. Hematologic changes following the administration of large doses of quinacrine hydrochloride *Blood* 1 537 1946
- <sup>22</sup> GRÜNEBERG H. Siderocytes in man *Nature* 148 469 1941
- <sup>23</sup> OTTO, T. O. AND REZEK P. A new type of Bartonella infection in man? *J Florida M. A* 34 64 1943
- <sup>24</sup> BETHELL F. H. STURGIS C. C. RUNDLES R. W., AND MEYERS M. C. Blood A review of recent literature *Arch Int Med* 76 358 1945
- <sup>25</sup> ROSENTHAL, N. Clinical and hematologic studies in Banti's disease *J A M. A* 84 1887 1925
- <sup>26</sup> EVANS W. H. The blood changes after splenectomy in splenic anaemia purpura haemorrhagica and acholuric jaundice with special reference to platelets and coagulation *J Path & Bact* 31 815 1928
- <sup>27</sup> DAWBARN R. Y. EARLAM F. AND EVANS W. H. The relation of the blood platelets to thrombosis after operation and parturition *J Path & Bact* 31 833 1928
- <sup>28</sup> ADAMS E. Post-operative thrombocytosis *Arch Int Med* 73 329 1944



TABLE I

Subject Number	RBC	Hgb	Hct	MCV	MCH	MCHC	Retic
<i>I Normal Group</i>							
8	5 1	14 2	42 5	84	28	33	1 5
9	5 5	16 2	49 2	89	29	33	8
10	5 1	15 5	46 0	90	31	34	6
58	4 8	14 1	44 2	91	29	32	8
87	5 6	16 0	46 5	82	23	34	1 3
91	5 5	15 7	44 0	80	29	36	1 6
92	4 9	15 1	44 0	88	31	34	9
93	5 1	15 8	45 5	88	30	33	1 1
94	4 8	14 6	44 0	92	30	33	1 5
<i>Miscellaneous</i>							
71	4 13	11 5	35 4	86	28	32	1 3
42	5 55	16 3	50 3	91	29	32	7
43	4 39	13 1	42 0	96	30	31	9
<i>II Iron Deficiency</i>							
20	2 48	7 4	24 5	99	30	30	17 3
5	3 1	8 6	26 1	84	28	33	3 2
19	2 68	8 7	28 3	106	33	31	2 2
26	3 48	6 3	25 0	72	18	25	7 7
24	2 92	8 5	28 7	98	29	30	5 3
7	3 33	5 3	24 4	73	16	22	2 1
<i>III Hemochromatosis</i>							
108	4 12	13 7	38 8	94	33	35	9
86	4 86	15 4	47 5	98	32	32	1 6
83	3 85	12 4	36 3	94	32	34	8
<i>IV Refractory Anemia</i>							
11	3 74	11 0	35 2	94	30	31	7 5
14	2 0	6 7	20 4	102	33	33	2 7
66	3 32	9 3	27 4	83	28	34	1 5
<i>V Uremia</i>							
29	4 2	12 5	39 7	95	30	31	1 0
41	2 13	7 2	22 8	107	34	31	5 1
62	3 35	11 1	32 5	97	33	34	2 0
15	2 55	6 2	21 0	82	26	32	2 1
14	2 28	6 3	20	88	28	32	2 1
<i>VI Infection</i>							
44	5 1	14 9	44 2	87	29	34	1 1
57	4 71	13 1	40 0	85	28	33	1 4
18	4 53	12 8	39 3	87	28	33	1 0
27	3 62	11 0	35 6	98	31	31	9
46	4 76	13 9	43 8	92	29	31	1 6
17	4 12	12 1	40 2	98	29	30	1 5
69	4 58	12 2	40 0	87	27	30	
47	3 67	9 9	33 8	97	27	29	3 3

TABLE 1—*Continued*

Subject Number	RBC	Hgb	Hct	MCV	MCH	MCHC	Retic
<i>VII Hemolytic Anemia</i>							
40	97	3.7	12.8	132	40	29	38.0
52	129	8.0	24.3	98	32	33	3.6
2	332	9.8	31.0	93	29	31	7.0
75	181	5.8	19.5	108	32	30	8.6
	99	3.6	10.5	106	36	34	1.0
<i>VIII Pernicious Anemia</i>							
49	67	2.9	9	34	43	32.2	4
55	148	5.0	16.2	109	34	31	2.0
53	314	10	30.1	96	31.9	33.2	1.1
<i>IX. Malaria</i>							
28	43	11.9	38.8	90	28	31	5
51	349	10.5	35	100	31	31	
64	477	13.0	42	88	27	31	7
80 (IC)			42				
<i>X Malignancy</i>							
45	35	9.2	30.7	88	27	31	1.1
56	416	10.7	34.6	83	26	31	1.4
<i>XI Hepatic Disease</i>							
12	335	11.7	35.5	106	35	33	4.5
34	252	7.5	25.8	102	30	29	1.6
6	447	13.3	39.1	87	30	34	1.7
<i>XII Endocrinological Disease</i>							
54	30	9.8	31	103	32	33	
23	354	9.6	30.5	86	27	32	2.6
35	48	14.1	42.3	88	29	33	1.7
33	407	11.5	35.9	88	28	32	1.1
25	392	10.5	32.9	84	27	32	2.1
22	458	13.7	43.5	95	30	31	1.0
<i>XIII Polycythemia</i>							
63	55	14.8	49.0	88	27	30	1.0
31	782	16.4	58.1	74	21	28	1.5

metal into compounds suited for intravenous injection the preparation of blood samples for radioactivity measurement and the use of differential counters for the simultaneous measurement of  $\text{Fe}^{55}$  (by x-rays) and of  $\text{Fe}^{59}$  (by beta ray) have been described by Peacock et al.<sup>8</sup> For most of the experiments  $\text{Fe}^{55}$  (half-life four years) was used. Various acidified salts were employed including ferric chloride, ferric-ammonium citrate, and ferrous ammonium sulfate. Carrier iron had been added to bring the total iron content injected to 0.1 and 0.5 mg. and each injection contained approximately one million counts per minute. As far as could be determined, these compounds were handled in identical fashion in the body when given intravenously. Over a period of two to three weeks after the injection of radioiron, samples of venous



blood were obtained in the morning the patient was fasting in most instances. Hematologic studies were done according to the following methods. Hematocrit determinations were performed in Wintrobe tubes with centrifugation for one hour at 3 000 r p m (International Centrifuge Size I Type C) hemoglobin was determined in duplicate by the oxyhemoglobin method on an Evelyn colorimeter<sup>9</sup> red counts were done in duplicate pipets and were repeated if they did not check within 5 per cent. Reticulocyte counts were done according to the method of Osgood and Wilhelm.<sup>10</sup> Cell constants were determined and reticulocyte counts were performed at least twice during the study of each patient. In all patients whose blood picture was stabilized during the period of study the figures were averaged in table 1 for the sake of brevity. In the others blood values at the initiation of the study are recorded.\* Bilirubin determinations were made according to the method of Evelyn and Malloy.<sup>11</sup> Blood volumes were determined by the method of Gibson and Evans.<sup>12</sup> Four to six samples of blood were drawn between ten to thirty minutes after injection of the dye and read in the Evelyn photo-electric microcolorimeter. The circulating red cell volume was taken as 85 per cent of the cell volume calculated from the plasma volume and venous hematocrit.<sup>13</sup> The radioactivity present in the blood stream which was solely intracellular after the first day was expressed as per cent utilization of the total quantity given according to the formula: Per cent utilization =  $\frac{(\text{counts per minute/cc red cells}) \times (\text{red cell volume})}{\text{Total counts/minute injected}}$ . Since a dilution of the iron injected was run with the samples obtained from the patient decay in radioactivity and variation in counting efficiency were automatically corrected.

## EXPERIMENTAL DATA

### I Normal subjects (Nos 8, 9, 10, 58, 87, 91, 93, 94)

Nine normal male volunteers between the ages of 24 and 30 were used as subjects. None had recently given blood, or suffered any other blood loss. Blood volumes were determined at the beginning and in five instances at the end of the experimental period. Hematologic data are recorded in table 1. Utilization of intravenously injected radioiron is recorded in figure 1. Over a period of fifteen to eighteen days, 8 of these subjects showed a utilization of between 68 and 83 per cent, averaging 74 per cent †.

Three subjects (71, 42, 43) with miscellaneous diseases not expected to alter iron metabolism were studied in a similar manner. These included a 59 year old female diabetic (71) recovering from mild diabetic acidosis, a 53 year old male with typical myocardial infarction (42) but without any fever or evidence of heart failure during the period of study, and a 71 year old asthmatic (93) in no acute distress. Their utilization curves shown in figure 2 were similar to the composite curve of normal subjects.

### II Iron Deficiency and Blood Loss Anemia (5, 7, 19, 20, 24, 26)

Six patients with acute or chronic blood loss were given radioiron ( $\text{Fe}^{55}$ ) intravenously. The patients represented varying degrees of iron deficiency as shown in table 1 by their degree of microcytosis and hypochromia. Slight increases in mean cell size found in acute blood loss are undoubtedly due to the appearance of younger cells which are larger. Some patients had continued bleeding, some were

In the reprints of this article charts are included portraying the clinical course of these patients similar to those shown in figure 7. While a correlation of the clinical factors affecting erythropoiesis and the utilization curve was thought to be important space did not permit its inclusion in the Journal.

† Subject 93 was excluded because of his variation from the others and because of the finding of a decreased amount of iron binding protein in his serum.

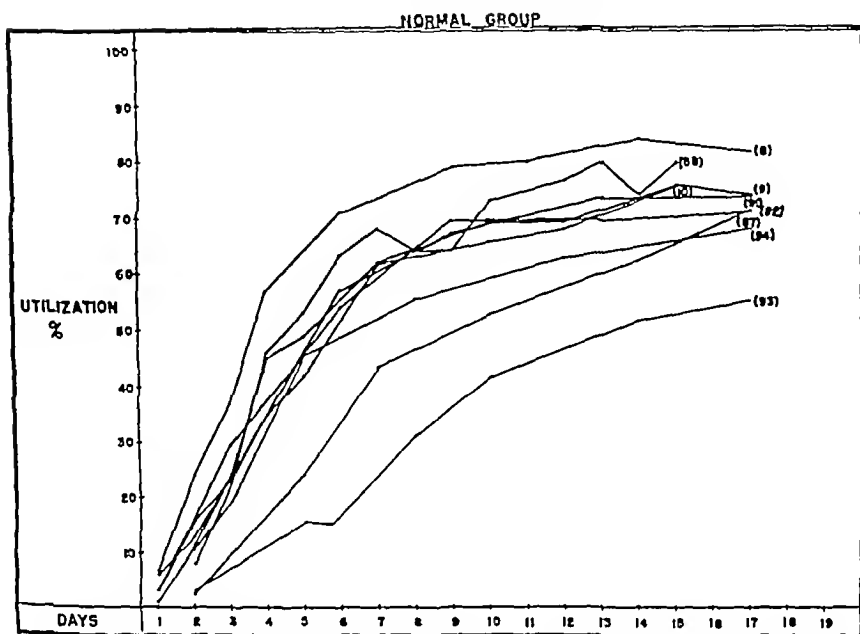


FIG 1

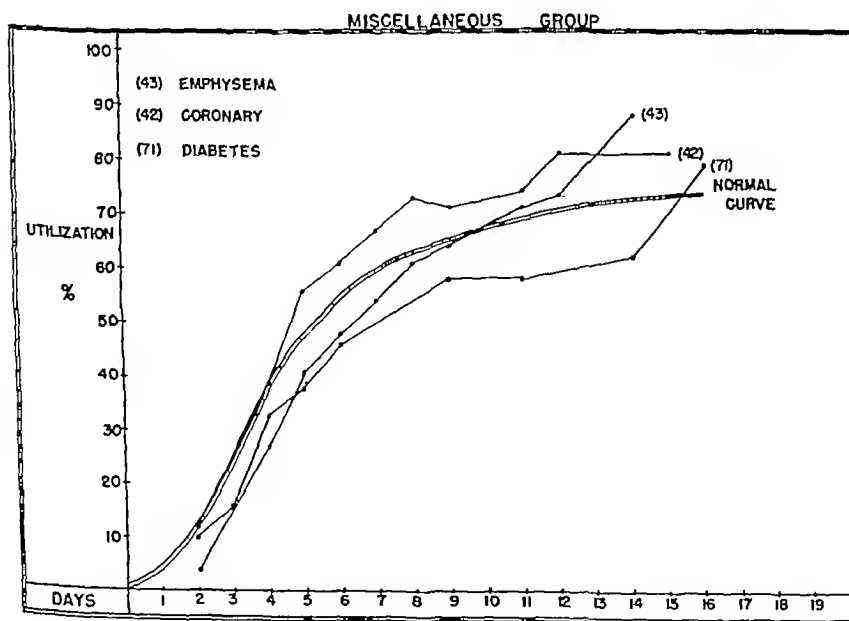


FIG 2

given iron therapy, some showed limited red cell production due to iron lack, while in others red cell regeneration was rapid. Patients 7, 19 and 26 showed a fall in the utilization curve during the second week. This may be related to changes in the total blood volume, since only an initial blood volume determination was made and subsequent changes in cell mass were calculated from the hematocrit.

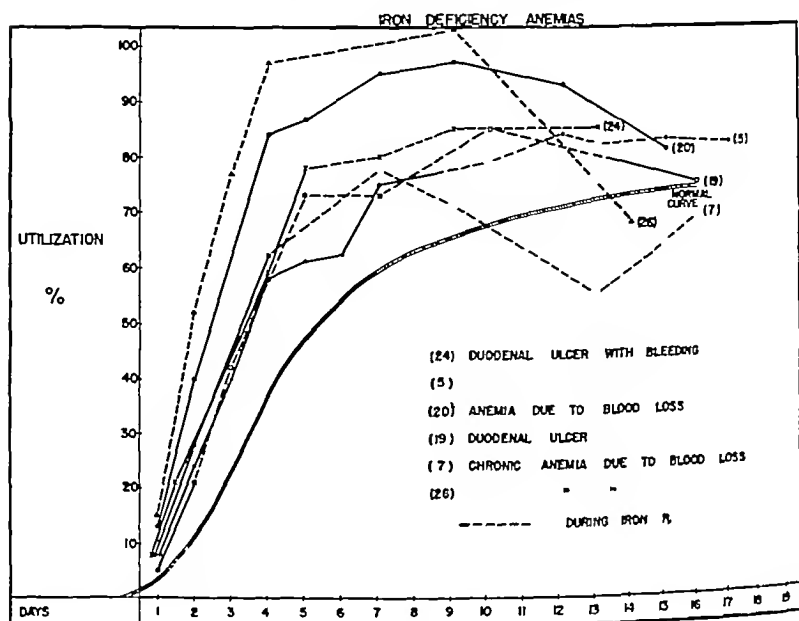


FIG 3

It might also be explained by continued blood loss. All patients, however, showed a rapid utilization of the injected radio-iron (fig 3).

### III Hemochromatosis (83, 86, 108)

Three patients with hemochromatosis confirmed by liver biopsy were studied. While 2 patients had slight anemia and microcytosis, patient 86 had normal blood values (Table 1). The radioiron utilization curve of all patients was depressed (fig 4) in the presence of fairly normal red cell production.

### IV Refractory, Aplastic, and Myelophthisic Anemia (11, 14, 66)

Three different types of bone marrow dysfunction were studied. Patient 11 was a 22 year old woman with refractory anemia and a hyperplastic bone marrow. Patient 14, had acute disseminated lupus erythematosus with an aplastic marrow at post mortem examination and Patient 66, had extensive lymphosarcomatous involvement of the bone marrow. In these cases only small amounts of radioactivity appeared in the peripheral blood (Fig 5).

## HEMOCHROMATOSIS

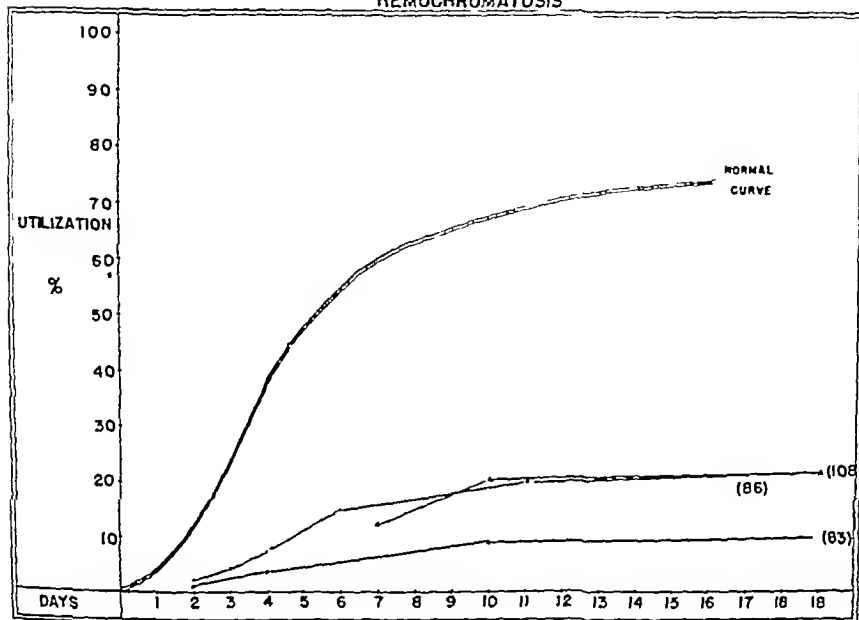


FIG 4

## REFRACTORY APLASTIC AND MYELOPHTHISIC ANEMIAS

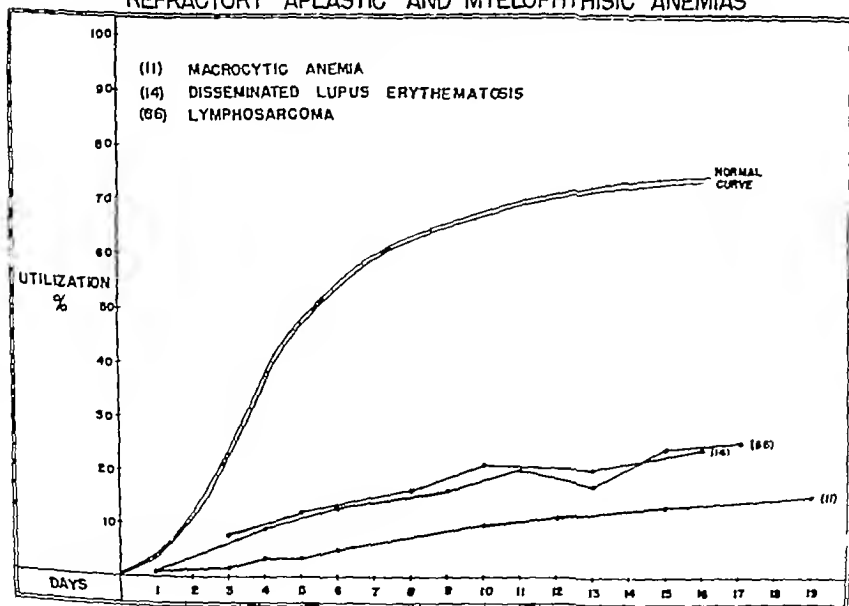


FIG 5

### V Uremia (4, 15, 29, 41, 62)

The five cases of renal disease included one patient with acute glomerulonephritis (29), 2 patients with lower nephron damage (41, 62) and 2 with chronic nephritis without edema (15, 4). In Patients 4, 15, and 41 blood transfusions had previously been given. All showed some degree of anemia (table 1), thought to be due to the uremic state, except for 41, where severe hemolysis had resulted in both renal damage and anemia. Utilization curves (fig. 6) were all depressed below normal.

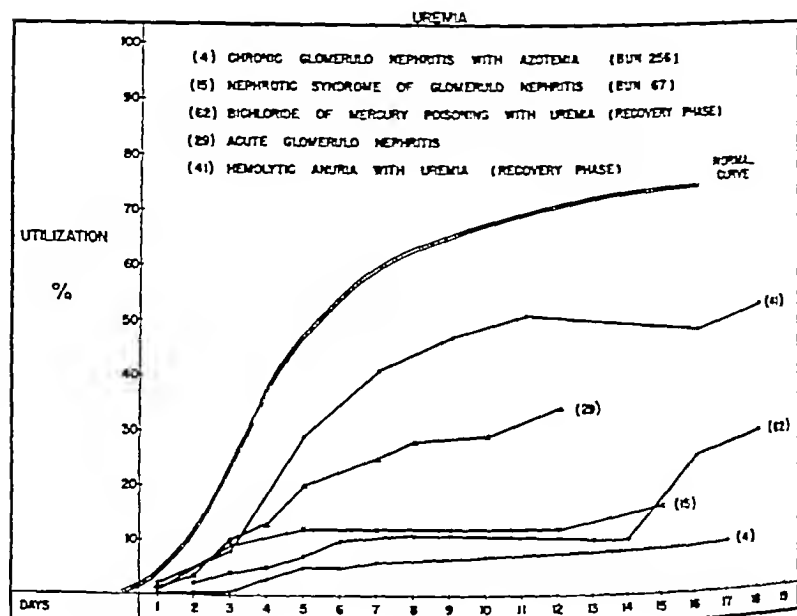


FIG 6

### VI Infection (17, 18, 27, 44, 48, 57, 69)

Eight patients with infection of viral, bacterial, and protozoal etiology were studied. These infections were of variable duration. 44, 57, 27, and 17 were of less than two weeks duration. 69 and 46 of about one month, and the two patients with subacute bacterial endocarditis (18 and 47) of several months duration. In figure 7 are shown the clinical course of a patient with a mild viral pneumonitis and a patient with severe pneumococcal pneumonia. Iron utilization curves, shown in Figures 8 and 9 are extremely depressed in the severely ill patients.

### VII Hemolytic Anemia (2, 3, 52, 40, 75)

One patient with sickle cell anemia (2), one with congenital hemolytic anemia (3) and three with acquired hemolytic anemia were studied. Several of these

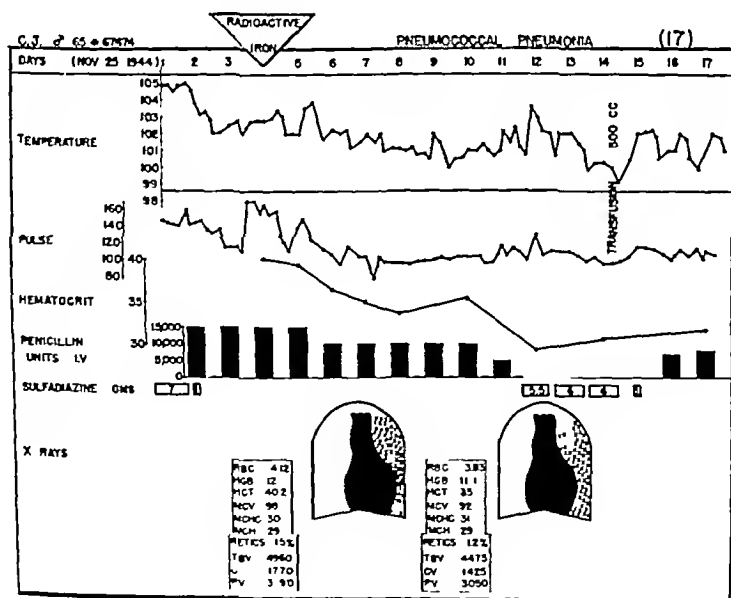
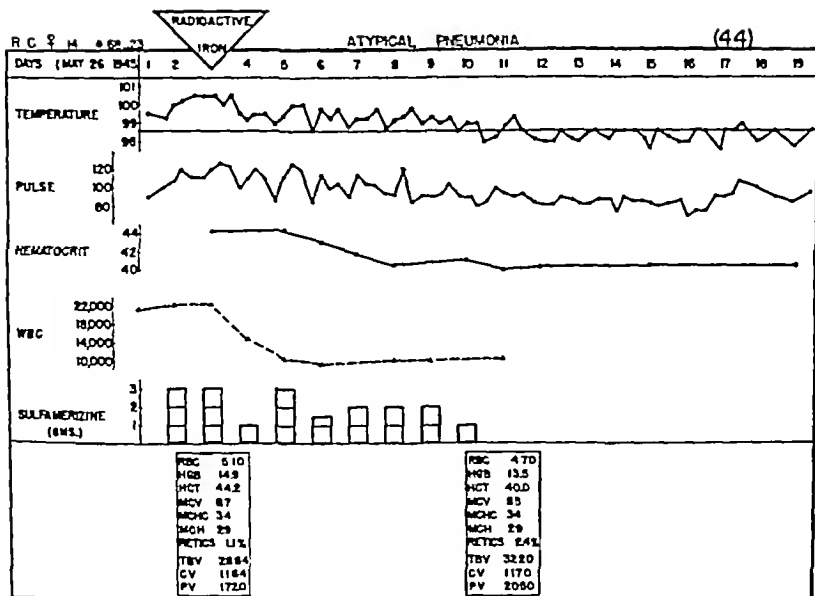


FIG 7

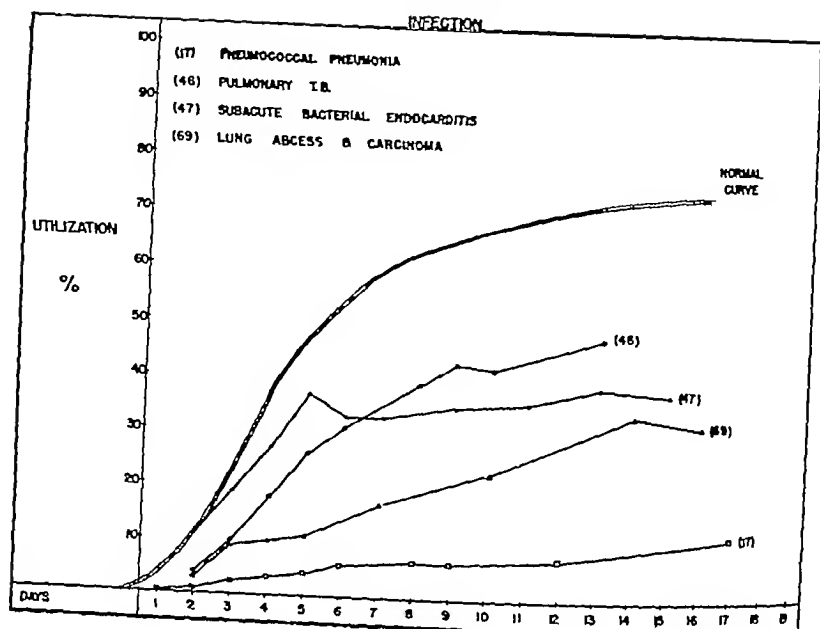


FIG 8

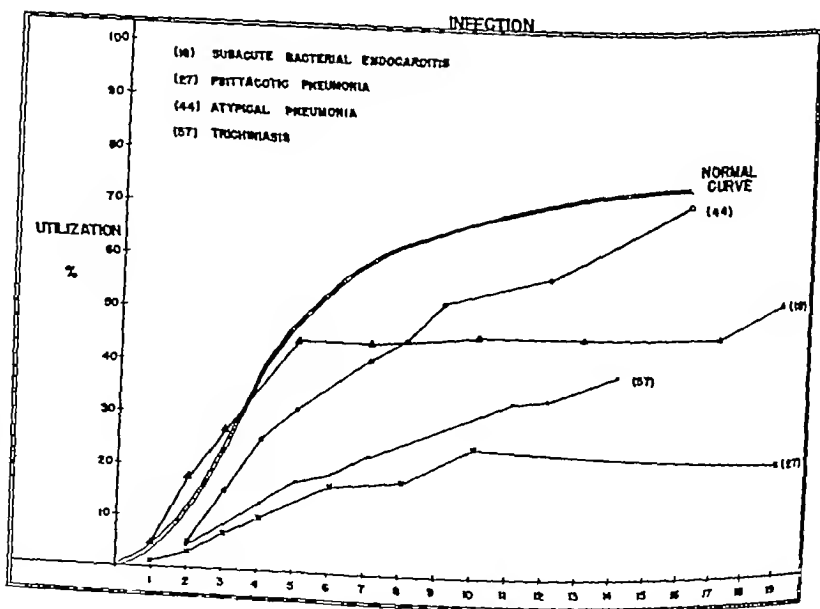


FIG 9

patients had been repeatedly transfused before the radioiron was injected (75), or were given blood during the period of study (40, 52). In only the patient with sickle cell anemia was there no blood administration. All cases showed a rapid initial rate of utilization and maximum values were obtained in three to five days (fig. 10). However, the total amount in circulation was very low. The utilization curve of Patient 3 was repeated one year after her hemolytic episode at a time when her peripheral blood picture was normal (fig. 11). In the interim she had lost no blood other than the normal amount through menstruation. The only difference between the two utilization curves might be assumed to be the state of severe hemolysis during the first study.

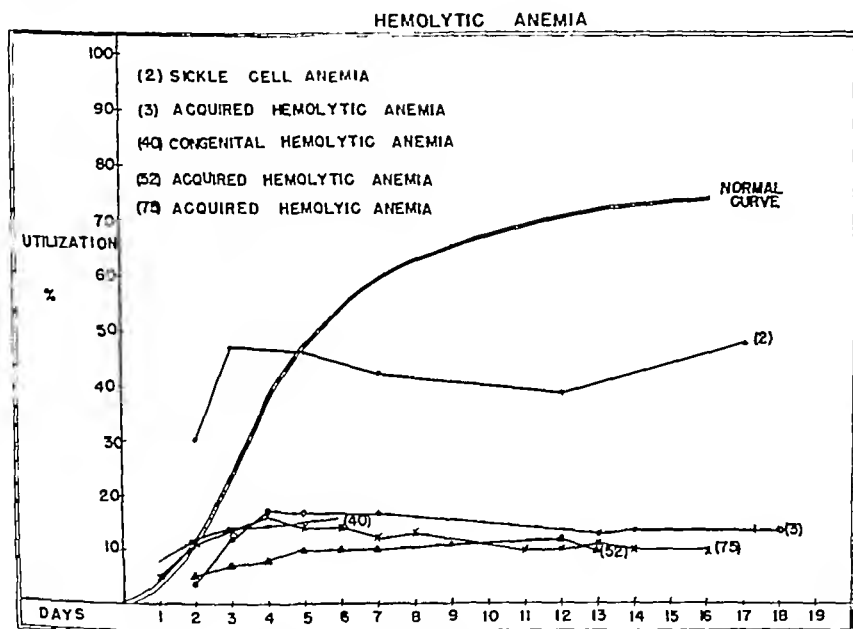


Fig 10

### VIII Pernicious Anemia (49, 53, 55)

Three patients with pernicious anemia were studied during a period of active blood production following liver therapy (fig. 12). In 53 and 55, the radioiron was given before liver therapy was effective, and the utilization retarded. In Case 49, however, iron administered several days after liver therapy was utilized rapidly and an early plateau was reached.

### IX Malaria (28, 51, 64, 80)

Radioiron utilization for hemoglobin synthesis was followed in 4 patients with paresis during a course of therapeutic malaria (*plasmodium vivax*). In figure 13,



## HEMOLYTIC ANEMIA

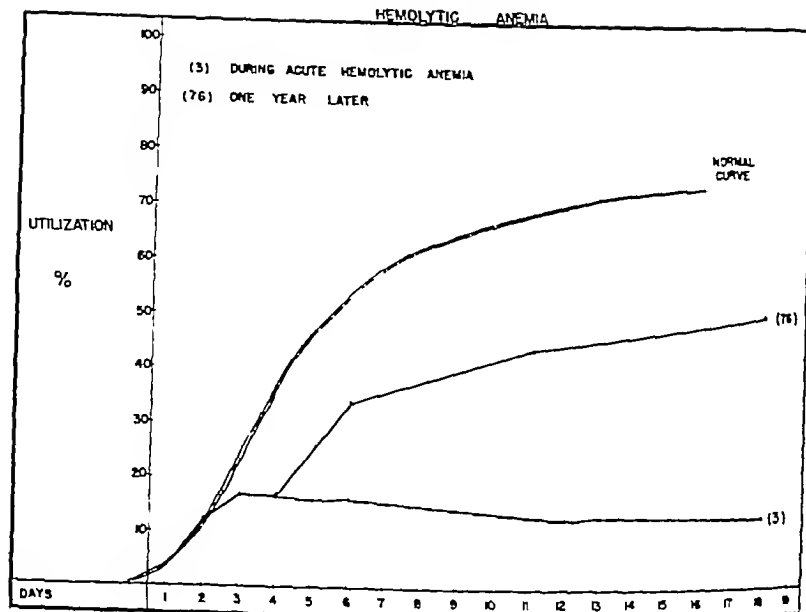


FIG 11

## PERNICIOUS ANEMIA

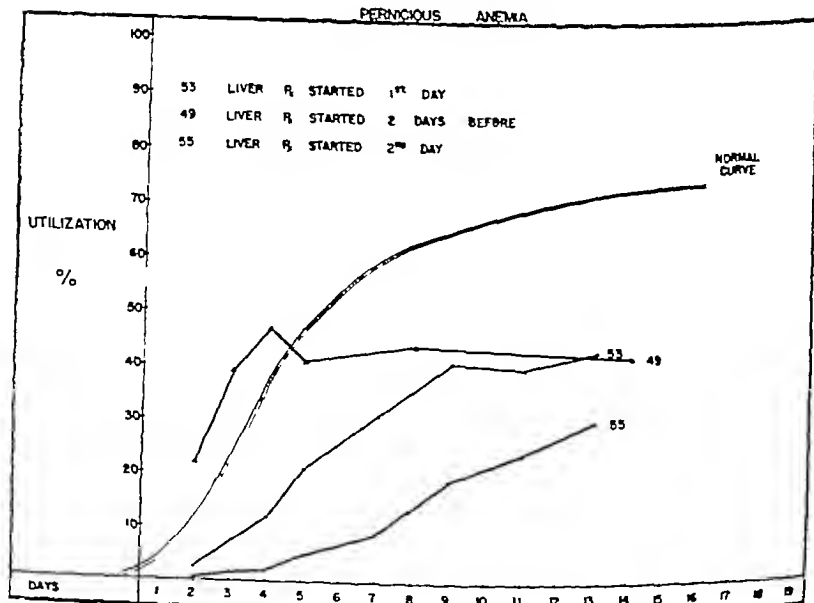


FIG 12

the dotted line represents periods of fever. It will be observed that the utilization of radioiron was markedly depressed during the malarial paroxysms and that in one instance the level of radioactivity fell (51). A fall in hematocrit also occurred in these patients during the active infection.

#### X Malignancy (45, 56)

Patient 45 was a 65 year old woman with probable adenocarcinoma of the left kidney and metastases to the right femur. Patient 56 was a 42 year old man

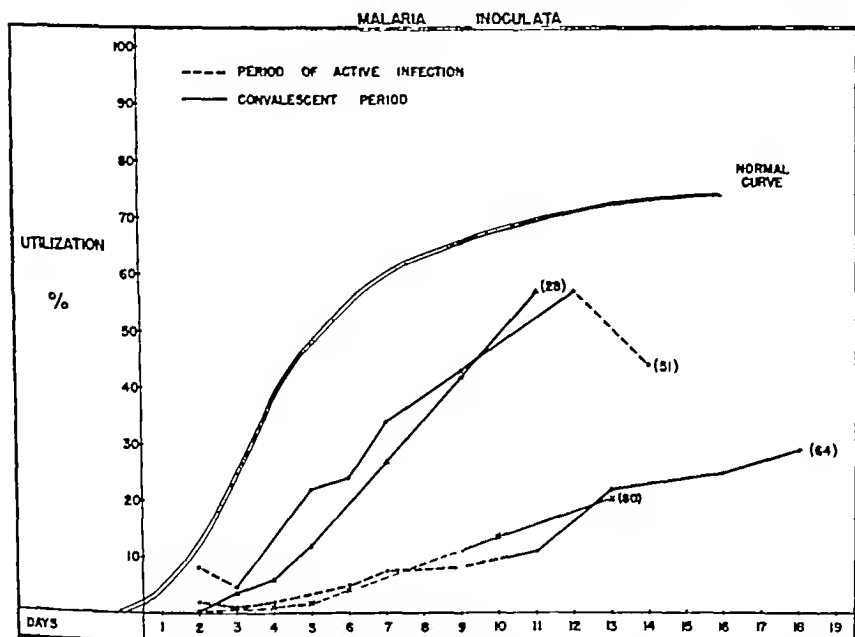


FIG 13

with a bronchogenic carcinoma, confirmed at autopsy. Both patients were afebrile and had no blood loss. Utilization curves were depressed (fig 14).

#### XI Endocrine disease (23, 25, 33, 35, 54)

Four patients with typical Addison's disease with a mild normocytic anemia were studied and utilization curves (fig 15) were found to be within normal range. A patient with postoperative myxedema (22) with a basal metabolism of  $-31$  also approximated normal utilization. However, patient 54, a 58 year old woman with anterior pituitary hypofunction showed a definite decrease in radioiron utilization (fig 16).

#### XII Miscellaneous (6, 12, 34, 31, 63)

A 23 year old girl with mild acute infectious hepatitis (6) showed normal utilization. A patient with Laennec's cirrhosis and obstructive jaundice (12) and

## MALIGNANT NEOPLASM

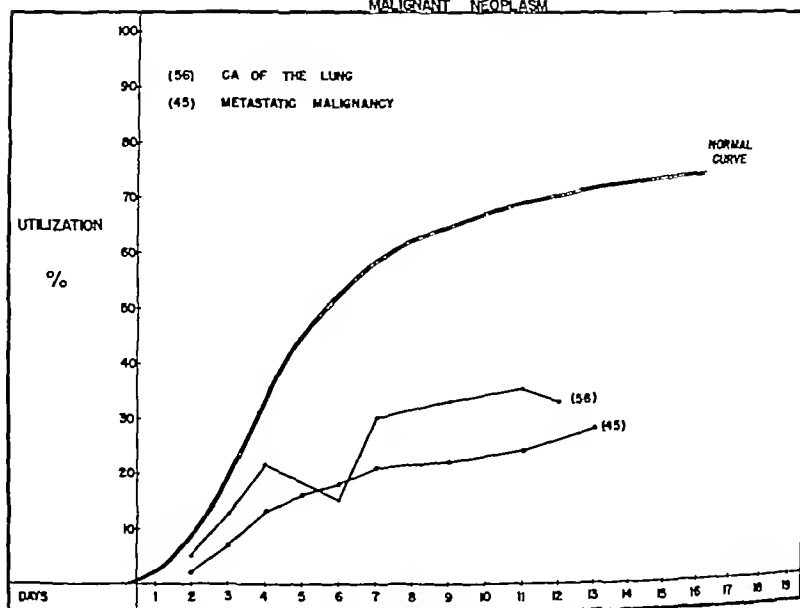


FIG 14

## ADDISON'S DISEASE

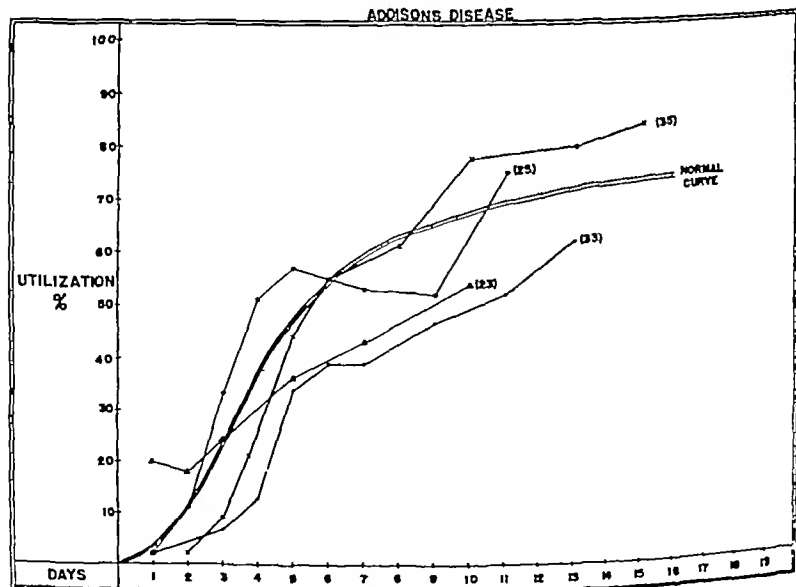


FIG 15

Patient 34, a 51 year old woman with toxic cirrhosis both showed utilization of 40-45 per cent at the end of eighteen days

A patient with polycythemia vera (31) who had been treated for two years by phlebotomies showed a rapid utilization to 90 per cent within the first week, while a patient with chronic congestive failure and secondary polycythemia (63), showed a normal utilization of iron for hemoglobin production

#### THYROID HYPOFUNCTION

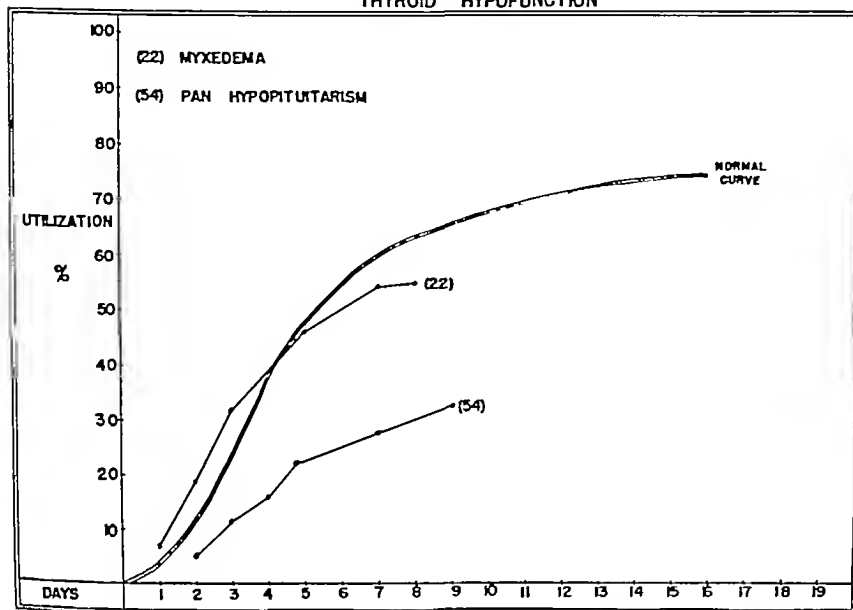


FIG 16

#### DISCUSSION

The preceding data represents the utilization of injected radioiron for hemoglobin production in man. Problems which are raised in this type of study include (1) the irradiation hazard of the isotope injected, particularly since it is not excreted from the body, (2) the exchange rate between hemoglobin iron and other body iron, (3) the nature of the utilization curve including the pathway taken by injected iron and factors influencing its synthesis into hemoglobin, (4) the interpretation of the utilization curve in the various pathological states investigated.

#### *Irradiation Hazard*

An approximation of the radiation produced by the injected iron can be made. The normal adult has a bone marrow space ranging from 1,600 to 3,700 cc averaging 2,600 cc.<sup>14</sup> It is likely that only about one-half of this marrow is active in the sense that it is engaged in the formation of erythrocytes and leukocytes. Only

about one-fifth of the active marrow space is concerned with erythropoiesis, the other four-fifths producing white cells in the myeloid series

Using these values, however approximate, one may estimate the radioiron of the bone marrow or any fraction thereof, in an individual receiving one million counts per minute, of radioactive iron. Our normal build-up curves show about 75 per cent localization of the injected activity within the red cell mass. Therefore, 25 per cent or  $2.5 \times 10^6$  will be found in the fixed tissues. This amount will be contained in liver, spleen, and bone marrow for the most part. Let us assume that all of this activity is distributed evenly within the bone marrow. In the case of  $\text{Fe}^{55}$ , the counter efficiency is 0.03, according to Peacock et al.<sup>3</sup> Therefore,

$$(1) \quad \frac{2.5 \times 10^6}{0.03} = 8.3 \times 10^7 \text{ disintegrations per minute in the whole bone marrow}$$

Divided by weight of marrow in grams

$$(2) \quad \frac{8.3 \times 10^7}{2.6 \times 10^3} = 3.2 \times 10^4 \text{ or}$$

$$(3) \quad 3.2 \times 10^4 \times 5.9 \times 10^5 = 1.88 \times 10^7 \text{ ev/Gm./min. or}$$

$$(4) \quad 1.88 \times 10^7 \times 1.44 \times 10^2 = 2.71 \times 10^{10} \text{ ev/Gm./day or}$$

$$(5) \quad \frac{2.71 \times 10^{10}}{52 \times 10^{12}} = 0.0005 \text{ roentgens equivalent physical per day for } \text{Fe}^{55}$$

if all the activity in active marrow = 0.001 r.p./day and if all activity in erythropoietic areas = 0.005 rep/day

Similarly the counter efficiency for  $\text{Fe}^{59}$  is 0.25. Therefore

$$(1) \quad \frac{2.5 \times 10^6}{0.25} = 1.0 \times 10^6 \text{ dis./min. in whole marrow}$$

Dividing by weight of marrow in grams

$$(2) \quad \frac{1.0 \times 10^6}{2.6 \times 10^3} = 3.85 \times 10^2 \text{ dis./Gm./min. or}$$

$$(3) \quad 3.85 \times 10^2 \times 0.12 \times 10^6 \text{ ev} = 4.62 \times 10^7 \text{ ev/Gm./min. or}$$

$$(4) \quad 4.62 \times 10^7 \times 1.44 \times 10^2 = 6.64 \times 10^{10} \text{ ev/Gm./day or}$$

$$(5) \quad \frac{6.64 \times 10^{10}}{52 \times 10^{12}} = 0.0013 \text{ roentgens equivalent physical per day for } \text{Fe}^{59} \text{ if all activity in erythropoietic areas} = 0.013 \text{ rep/day}$$

The above data indicate the upper level of activity in bone marrow due to extra circulating radioiron. Since it is known that much of the activity is stored in the liver and spleen, and if an even distribution is assumed in all three organs, the calculated bone marrow radiation dose given above for red cell forming marrow should be multiplied by 0.13.

It, therefore, seems probable that the radiation due to extracirculating radioiron in those fixed tissues containing the highest activity will not exceed 0.0008 r/day for  $\text{Fe}^{55}$ , and 0.002 rep/day for  $\text{Fe}^{59}$  in the normal adult male injected with one million counts per minute. The tissue irradiation is, therefore, calculated to be from 1/50th ( $\text{Fe}^{59}$ ) to 1/100th ( $\text{Fe}^{55}$ ) of the maximum permissible dose of 0.1 r (or rep) per day.

The amounts of iron injected in these experiments represent about one ten thousandth of the total body iron and after injection raised the serum iron less than

10γ/100 cc Thus the radioiron may be regarded as a true tracer dose of iron which would enter into the normal body iron turnover without altering it in any way

### *Exchange of Hemoglobin and Tissue Iron*

Iron gains access to the red cell only in its developmental stage, to be synthesized into hemoglobin Studies with reticulocytes have shown an active uptake of radioiron by these cells in vitro<sup>15</sup> When mature erythrocytes are incubated with radioiron, no uptake occurs When radioiron is injected into patients with little or no bone marrow function, little or no radioactivity appears in the red cell mass Once incorporated in the cell, the iron remains fixed there until the cell is destroyed In patients with large iron reserves it is possible to determine the life span of transfused tagged cells, since the iron liberated from senescent erythrocytes is diluted by the large reserve stores and only a small portion is reutilized Free exchange of iron does not occur, therefore, between erythrocytes and plasma or tissues

### *Nature of the Utilization Curve*

The amount of radioactivity entering the circulation over a period of fifteen to twenty days is a composite of three interdependent parts of iron metabolism the serum iron transport mechanism, the size and availability of iron stores, and bone marrow function

Within ten minutes after injection, one-third to one-fourth of the radioactivity has disappeared from circulation and the remainder clears exponentially from the serum, 50 per cent in about one and one-half hours<sup>16</sup> This latter fraction is bound in the plasma to a B<sub>1</sub> globulin which functions as a transport protein for iron<sup>17</sup> The amount of injected radioiron initially bound to this protein is fairly constant, unless the protein is already saturated with iron

Much of the radioiron may be found in the liver within a few hours after injection<sup>18</sup> Granick and Hahn have found this to be in the form of ferritin iron which probably represents the more labile form of storage iron The radioiron is then rapidly rerouted to the bone marrow for hemoglobin production About half of the total utilization of radioiron for hemoglobin production occurs over a period of 2.2 days Assuming that this radioiron was first mixed with tissue stores and then carried to the bone marrow, we may calculate the reserve iron to be about 100 mg Storage iron in man is considerably greater than this, therefore, radioiron can not completely mix with iron in storage This has led to the postulation of a very small labile iron reserve\* It seems more in harmony with our observations to think of this not as a special form of storage iron, but to postulate that the iron has fallen on the topsoil of iron stores which would be more labile from a physical standpoint as suggested by Dubach, Moore, and Minnick<sup>7</sup>

\* Red cell life span has been established in man at about 120 days Therefore 0.83 per cent of blood is broken down and rebuilt each day In a blood volume of 5 000 cc containing about 2,500 mg of iron this amounts to 21 mg of iron If radioiron labels a small active compartment 50 per cent of which turns over every 2.2 days the compartment size to the first approximation would be 92 mg

Over the days following injection, the radioiron passes through the serum to the bone marrow. The functional integrity of the erythropoietic tissue is the final link in the incorporation of the radioiron into the red cell.

The curve of radioiron utilization for hemoglobin production in normal subjects is approximately exponential in character. When normalized to 100 per cent, this curve extrapolates to a theoretic lag period of 1.8 days. Actually there is an appreciable uptake during this two day period, considerably greater in certain pathologic states. This is at least partially explained by the observation that reticulocytes in vitro will take up radioiron. It might be presumed that the reticulocytes in the circulation and the cells just leaving the marrow would begin to assimilate radioiron immediately after its injection. In addition, the composite normal curve is derived from the numerical average of eight subjects and shows a straggling effect which in part explains this initial rise in the utilization curve.

#### *Interpretation of the Utilization Curve*

In interpretation of the utilization curve there are two components of importance: the size of iron stores, and bone marrow function. The influence of enlarged iron stores was demonstrated experimentally in dogs (table 3). Utilization curves done after iron injections showed marked depression although erythropoiesis was unaffected. A similar reduction in utilization has been produced in experimental subjects by oral ingestion of iron over a period of six months. This again occurred without change in peripheral blood or in serum iron levels. The effect of bone marrow dysfunction is self evident from previous discussion.

The normal curve shows the localization of about 25 per cent of the radioiron extravascularly and 75 per cent in circulation as hemoglobin. This pattern may be taken as representative of the average storage iron compartment size and normal bone marrow function. The extravascular iron admittedly represents iron incorporated in cell enzymes and myoglobin as well as storage iron. With a decrease in storage iron as in iron deficiency, there may be increased demands of tissue for iron. This would make it unlikely that decreased iron stores would be accurately detected by the per cent utilization of radioiron. In conditions of iron excess, however, it seems definite that increased stores have a clear-cut effect in depressing utilization of radioiron for hemoglobin production.

In *iron deficiency and blood loss anemia*, initial utilization is more rapid and complete than in normal subjects. Comparing the curve in figure 3, it will be seen that Patients 20 and 26 show a greater utilization than the others. This is explainable on the basis of a greater bone marrow activity in these cases, one showing a reticulocytosis of 17 per cent, and the other responding with a rise in hematocrit to iron therapy. It is of some interest that blood production in patients 25, 5, and 7 was not increased during the first week, as judged by the hematocrit. With the hyperplastic marrow found in iron deficiency and therefore an increase in total red cell elements, the cell turnover would be slower than normal. The iron given, 0 to 0.5 mg, as compared with a daily breakdown and reutilization of about 20 mg, would not be expected to accelerate cell production. We must conclude that the increased speed of utilization in these cases represents a decrease in

storage, and in serum turnover time and perhaps a slightly shorter period of hemoglobinization of the red cell in the marrow. As the rate of erythropoiesis is increased by supplying more building materials, the utilization is further accelerated. It is of interest that 100 per cent utilization is not attained, suggesting that certain tissue requirements are met even with anemia. In *hemochromatosis*, radioiron utilization is profoundly depressed, while there is nothing fundamentally wrong with erythrocyte production. This clearly indicates, as did animal experiments (table 2), that radioiron to some extent measures tissue iron stores in that its utilization is inversely proportional to their size.

TABLE 2.—*Iron Loading Experiment in Dogs*

	Initial utilization (Fe <sup>55</sup> )	Iron injected	Subsequent utilization (Fe <sup>55</sup> )
	per cent	mg	per cent
G	77	1500	24
S	75	4170	16

Fifteen kilogram mongrel dogs were given radioactive iron (Fe<sup>55</sup>) intravenously and its utilization followed for 15 days. Blood volume of Dog G was 1550 cc. with hematocrit of 48% blood volume of Dog S was 1350 cc. with hematocrit of 51%. Over the following three months iron was injected as iron ascorbate gelatin. Subsequent utilization curves were performed showing greatly depressed utilization. Autopsies of the animals showed large iron deposits throughout the reticuloendothelial system of both animals.

TABLE 3.—*Rate of Radioiron Utilization for Hemoglobin Production*

Condition	Average time to achieve 50% of the maximum utilization observed
	days
Hemolytic anemia	2
Iron deficiency anemia	3
Normal	4
Hemochromatosis	4
Infection	5 or more
Myelophthasic anemia	8 or more

In conditions associated with *bone marrow dysfunction* (refractory, aplastic and myelophthasic anemias), the amount of radioactive iron appearing in the circulation was considerably reduced and the utilization curve was flattened. This same pattern was present in *uremia* and the impairment in utilization was roughly proportional to the degree of azotemia. In some instances body iron stores had been altered by previous transfusions (patients 15, 4, 41). This was not enough to explain the depression observed, and as demonstrated in Patients 41 and 62, when nitrogen retention was alleviated, iron utilization was improved. This would suggest that some factor associated with retention of metabolic products interferes with blood production as measured by iron utilization. There would not appear to be an attendant disorder in iron metabolism here, as the serum iron is usually within normal limits in contrast to the marked depression observed in infection.



Among the eight patients with *infection* studied, there was no reason to believe that any difference in iron utilization existed attributable to the etiologic agent. Rather, the depression in utilization curves appeared to be proportionate to the general severity of the infection. The curves showed the same gradual daily increments characteristic of decreased red cell production with the exception of 2 patients (18 and 47). Both of these had subacute bacterial endocarditis with associated splenomegaly. The rapid initial rise and early plateau in their utilization curves are similar to the curves in hemolytic anemia and raise the question as to whether increased hemolysis may have been present. This depressed utilization of radioiron in infection associated with a profound lowering in serum iron has been described in experimental animals.<sup>18</sup> The patients with *hemolytic anemia* show a different type of curve. Maximum utilization is reached on an average by the fourth day in contrast to the more gradual plateau normally found. A second feature of interest is the extremely low utilization observed in most instances. Previous blood transfusions may have depressed the utilization to some extent. In only Patient 2 was the experimental period entirely free of blood administration. However, while larger iron stores are to be expected in hemolytic anemia, these do not begin to reach the size found in hemochromatosis. This would suggest that, in hemolytic anemia, the serum iron binding protein is almost completely saturated with iron from broken down erythrocytes with the result that the injected radioiron is at once deposited in inactive tissue stores. Figure 11 substantiates this, for the utilization curve during the acute hemolytic stage was only 15 per cent, while at a later date the utilization was 50 per cent. There was no reason to believe that iron stores had changed appreciably in the interim. This may indicate either that hemoglobin iron is necessarily used in preference to injected iron or that the transport mechanism was already saturated with iron and that the injected iron was therefore more rapidly taken out of circulation. Destruction of newly formed erythrocytes undoubtedly occurred in these patients. This would hasten the mixing of iron but would not necessarily effect the per cent utilization for hemoglobin production. *Pernicious anemia* presents a more complex situation. It will be observed that there are two types of curves. When the iron had opportunity to mix with the enlarged iron stores before liver therapy, its subsequent appearance in the circulation was slow. However, when the iron was given at a time when hematopoiesis was proceeding rapidly after liver therapy, utilization was rapid. This latter patient showed an early plateau, suggesting that by the fifth day there may be some destruction of the newly formed cells. Other observations on the viability of the reticulocytes in pernicious anemia<sup>16</sup> and studies<sup>20</sup> on the viability of erythrocytes in pernicious anemia substantiate this. In *malaria*, the decline in radioactivity in Case 51 indicates destruction of young cells in keeping with the previous observation that parasitized cells contain most of the radioactivity.<sup>21</sup> It will be observed that during the periods of fever there is little iron utilization, while after irradiation of the infection there is more rapid utilization.

Little is known of the mechanism of anemia in *malignancy*. The general contour of the utilization curve was fairly normal, but the utilization was less than half of normal. There was no evidence of hemolysis in these cases. It is impossible to

divorce the influence of storage size and bone marrow dysfunction here. It is reasonable to assume, however, that both may play a part. In keeping with the lack of any severe hematologic involvement in *Addison's disease*, the utilization curve was essentially normal. This was also true of mild myxedema. However, with the more severe anemia of panhypopituitarism, the utilization was depressed.

In figure 17, a diagrammatic representation of storage and circulating red cell iron in certain conditions was studied as compared with typical radioiron utilization

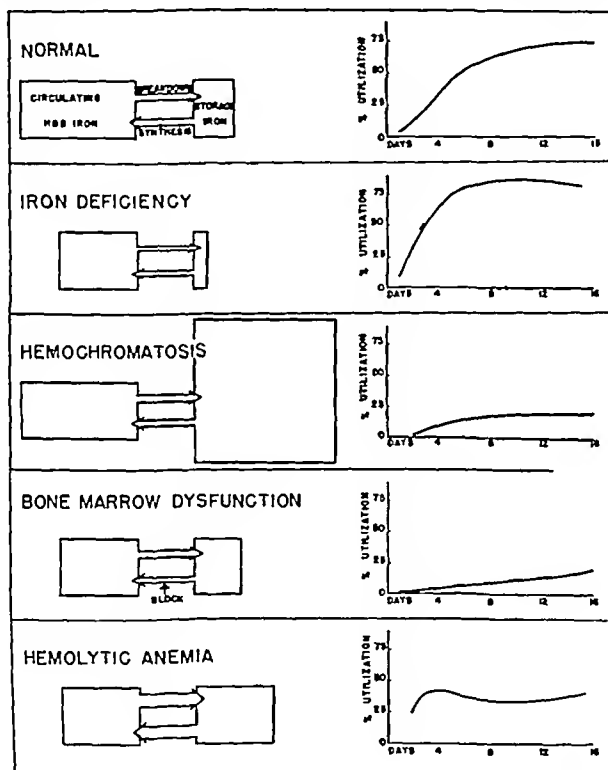


FIG 17

tion curves. In hemochromatosis and in iron deficiency, the primary factor influencing utilization was the size of iron stores. In hemolytic anemia and bone marrow dysfunction, the chief factor was rate of blood production in the bone marrow. It is of some interest that if the curves in both control subjects and in those patients with hemochromatosis are "normalized" to 100 per cent, their slopes are the same. In general, the slope appears to correlate with the rate of red cell production. As an index of this, the average time to reach 50 per cent of the utilization attained in two weeks is listed in table 3. The difference is somewhat

greater than apparent from the data when the period of lag is taken into consideration

These data are similar in general to the studies of iron utilization reported by Dubach, Moore, and Minnick.<sup>7</sup> Our lower utilization values may be accounted for in part by the assumption of blood volumes on the part of these authors without the additional correction factor of 0.85 per cent found necessary by Gibson et al.<sup>22</sup> Ross<sup>22</sup> finds a slightly lower normal utilization, in the neighborhood of 60 per cent. It seems likely that injected iron is used interchangeably with iron liberated from hemoglobin, for the utilization curves are quite similar from broken down hemoglobin and injected radioiron.<sup>2, 23</sup> The blocking action found in hemolytic anemias would appear to be due to the more saturated state of the serum iron binding protein forcing the injected iron into storage depots. In absorption studies employing radioiron, it is obvious, as previously suggested,<sup>7</sup> that the percentage utilization cannot be taken as the amount absorbed and that the studies of Hahn et al.<sup>24</sup> must be interpreted according to the expected utilization of iron, once this material gains access to the blood stream. The simultaneous intravenous injection and oral administration of different isotopes of radioiron might be expected to circumvent this.

#### SUMMARY

By determining the percentage utilization of intravenously administered radioiron for hemoglobin production over a period of two to three weeks, certain measurements of internal iron metabolism can be made.

With a normal rate of blood production, changes in per cent utilization reflect alteration in iron stores. Iron depletion is characterized by more rapid and more complete utilization of radioiron. States of iron excess in hemochromatosis can be identified by their profound depression of radioiron utilization.

If, on the other hand, storage iron is not greatly altered, the percentage utilization is determined by the function of the erythropoietic tissue. In myelophthisic anemias, in uremia, and in infection, a similar depression of the curve is found.

The rate of erythropoiesis may further be estimated by the slope of the utilization curve, and evidence of abnormal red cell destruction is found in early and abrupt plateau of the utilization curve.

A correlation has been made in a variety of hematologic disorders between the radioiron utilization for hemoglobin production and the clinical factors which might be expected to affect iron metabolism in these patients.

#### REFERENCES

- <sup>1</sup> McCANCE R. A. AND WIDDOWSON E. M. Absorption and excretion of iron following oral and intravenous administration. *J. Physiol.* **94**, 148-154, 1938.
- LITTLE, A. G. POWER M. H. AND WAKEFIELD E. G. Absorption and excretion of iron. *Ann. Int. Med.* **23**, 627-633, 1945.
- <sup>2</sup> McCANCE R. A. AND WIDDOWSON E. M. Iron excretion and metabolism in man. *Nature* **152**, 326-327, 1943.
- <sup>4</sup> GIBSON J. G. II, SELIGMAN A. M., PEACOCK, W. C., AUB J. C., FINE J. AND EVANS R. D. The distribution of red cells and plasma in large and minute vessels of the normal dog determined by radioactive isotopes of iron and iodine. *J. Clin. Investigation* **25**, 848-857, 1946.

- <sup>5</sup> HAHN, P F The metabolism of iron *Medicine* 16 249-266 1937
- <sup>6</sup> — WHIPPLE, G H II Iron metabolism, its absorption, storage, and utilization in experimental anemia *Am J M. Sc* 191 24 1943
- <sup>7</sup> DUBACH R MOORE C V, MINNICH V Studies in Iron Transportation and Metabolism V Utilization of intravenously injected radioactive iron for hemoglobin synthesis and an evaluation of the radioactive iron method of studying iron absorption *J Lab & Clin Med* 31 1201-1222, 1946
- <sup>8</sup> PEACOCK, W C EVANS R D, IRVINE J W, GOOD W M, AJP A F WEISS S AND GIBSON J G 2nd The use of two radioactive isotopes of iron as tracer studies of erythrocytes *J Exper Med* 25 605-615 1946
- <sup>9</sup> EVELYN K A Stabilized photo-electric colorimeter with light filters *J Biol Chem* 115 63-75 1936
- <sup>10</sup> OSGOOD E. E. AND WILHELM M M Reticulocytes *J Lab & Clin Med* 19 1129-1135 1934
- <sup>11</sup> MALLOY H T, AND EVELYN K A Determination of bilirubin with photoelectric colorimeter *J Biol Chem* 119 481-490 1937
- <sup>12</sup> GIBSON, J G 2nd EVANS W A Jr Clinical studies of blood volume Clinical application of method employing Azo dye Evans blue and spectrophotometer *J Clin Investigation* 16 301-316 1937
- <sup>13</sup> —, WEISS, S EVANS R. D PEACOCK, W C IRVINE J W GOOD W M, AJP A F The measurement of circulating red cell volume by means of two radioactive isotopes of iron *J Clin Investigation* 25 616-626 1946
- <sup>14</sup> MECHANICK, N Untersuchungen über das Gewicht des Knochenmarkes des Menschen *Ztschr Ges Anat* 79 58 1926
- <sup>15</sup> RATH, C E FINCH C A KOLLER F, GIBSON J G 2nd FLUHARTY R G Observations on reticulocytes To be published
- <sup>16</sup> — — GIBSON J G II FLUHARTY R. G Iron transport II Turnover rate To be published
- <sup>17</sup> RATH C E, AND FINCH, C A. Chemical, clinical and immunological studies on the products of human plasma fractionation XXXVIII, Serum iron transport measurement of iron binding capacity of serum in man *J Clin Investigation* 28 79-85 1949
- <sup>18</sup> GRANICK S AND HAHN P F FETTING VIII Speed of uptake of iron by the liver and its conversion to ferritin iron *J Biol Chem* 155 661-669 1944
- <sup>19</sup> GREENBERG G R ASHENBRUCKER, H LAURITSEN M. WORTH W HUMPHREYS, S R. WINTROBE M. M The anemia of infection V Fate of injected radioactive iron in the presence of inflammation *J Clin Investigation* 26 121-125 1947
- <sup>20</sup> CALLENDER, S T LOUITY J F AND JOPE E M. Discussion on the life and death of the red blood corpuscle *Proc Roy Soc Med* 39 755-762, 1946
- <sup>21</sup> FERREBEE J W GIBSON J G 2ND AND PEACOCK, W C Studies on malarial parasites IV Some observations regarding the age of the erythrocyte invaded by plasmodium vivax *J Infect Dis* 78 180-182, 1946
- <sup>22</sup> ROSS J Personal communication
- <sup>23</sup> GIBSON J G 2ND AUB J C EVANS R. D PEACOCK, W C IRVINE J W SACK, T The measurement of post transfusion survival of preserved stored human erythrocytes by means of two isotopes of radioactive iron *J Clin Investigation* 26 704-714 1947
- <sup>24</sup> BALFOUR W M HAHN P F BALE, W F POMMERENKE W T, AND WHIPPLE G W Radioactive iron absorption in clinical conditions Normal pregnancy anemia and hemochromatosis *J Exper Med* 76 15-30 1942.

# HEMOLYSINS IN ACQUIRED HEMOLYTIC ANEMIA

## EFFECT OF pH ON THE ACTIVITY IN VITRO OF A SERUM HEMOLYSIN

By J. V. DACIE, M.B., M.R.C.P. LONDON

**H**YDROGEN ion concentration has a controlling effect upon many hemolytic systems, both simple and complex. Osborn<sup>1</sup> in 1934 reviewed the early literature on the effect of pH on the hemolysis by complement of corpuscles sensitized by hemolytic immune body. He found that the optimum reaction for the hemolysis of sheep corpuscles by guinea pig serum was about pH 7.5 with inhibition below pH 5.5 and above pH 9.7. More recently, Seifter et al.<sup>2</sup> have reported unimpaired activity of human complement between pH 6.1 and 8.4 and irreversible and rapid destruction below pH 4.2 and above pH 10.1.

The effect of pH or carbon dioxide concentration on the activity in vitro of hemolytic antibodies of human origin has seldom been considered except in the case of chronic hemolytic anemia with nocturnal hemoglobinuria (Ham,<sup>3</sup> Dacie and Richardson<sup>4</sup>), in cold hemoglobinuria where the adjuvant effect of carbon dioxide on hemolysis has been sometimes referred to (Van den Bergh,<sup>5</sup> Hannema and Rytma,<sup>6</sup> Wagley, Zinkham and Siebens<sup>7</sup>) and in a case of acute hemolytic anemia in infancy reported by David and Minot.<sup>8</sup>

In the present communication are reported observations on the activity in vitro of an abnormal hemolysin in the serum of a patient with idiopathic acquired hemolytic anemia, and the effect of pH on its action. It was found that although little or no hemolysis resulted when normal Group O corpuscles were suspended in unacidified patient's serum (pH 8.0), hemolysis readily took place if the pH of the serum-corpuscle suspension was adjusted to an optimum (pH 6.8 to 7.0) by the addition of suitable volumes of acid. If graded amounts of acid were added to serum it could be shown that the range of pH within which hemolysis could be observed corresponded quite closely to that found in chronic hemolytic anemia with nocturnal hemoglobinuria (Dacie and Richardson<sup>4</sup>). In the final section of this paper, these observations are contrasted with the pH ranges for the hemolysis by complement of erythrocytes sensitized by anti-A or anti-B isohemolysin and of group O erythrocytes sensitized by a cold hemolysin present in the serum of a patient with cold hemoglobinuria.

### GENERAL TECHNICAL METHODS\*

Serum was obtained by defibrinating blood around a roughened glass rod in a conical flask. The pH of the serum was approximately 8.0. Serum intended as a source of complement was used within three hours of collection and stored frozen until utilized.

The pH of the serum was modified by the addition of 10 per cent by volume of N/5, N/10 or N/20 NaOH or N/20, N/10, N/5, N/4, N/3, 5 N/3 or N/2.5 HCl.

From the Department of Pathology, Postgraduate Medical School of London, London, England.

\* Other technical details are given in footnotes to table 1.

*Erythrocyte suspensions* were prepared from packed saline washed corpuscles and used at final concentrations of 2 per cent or 5 per cent by adding to the (acidified) serum 10 per cent by volume of a 20 per cent or 50 per cent suspension

*Serum-corpuscle suspensions* were generally incubated in a water bath at 37 C for 30 minutes

*Hemolysis* was measured photoelectrically after diluting in N/200 NaOH volumes of the supernatants obtained after centrifuging the corpuscle serum suspensions

The pH of the corpuscle serum suspensions was measured by a glass electrode at the end of the period of incubation and after resuspending the corpuscles or when only small volumes of serum were available by the use of indicators (phenol red bromthymol blue or methyl red)

## SUMMARY OF CLINICAL HISTORY AND ROUTINE LABORATORY FINDINGS

*Miss B, aged 18 Idiopathic acquired hemolytic anemia*

Splenectomy had been performed for hemolytic anemia approximately five years before the present series of observations was made The cause of the original hemolytic attack was uncertain, the family history did not suggest a familial incidence

The patient was admitted into a hospital in London in December 1946, severely ill with signs of intense hemolysis Repeated blood examinations then revealed a severe macrocytic anemia, the erythrocyte count was 10 to 15 million per cu mm, with 35 to 55 Gm hemoglobin, there was a high reticulocytosis (25 to 50 per cent) and a raised mean corpuscular volume (up to 160 cu micra) In films of peripheral blood there were occasional normoblasts, much polychromasia and postsplenectomy basophilic stippling, and rarely instances of erythrophagocytosis by mononuclear cells The Coombs test was positive and cold autohemagglutinins were present to a titer of 1:512 at 2 C, there was just perceptible autohemagglutination at 37 C The plasma bilirubin level was continuously raised (up to 4 mg per 100 ml) and there was a slight increase in plasma globulin (albumin 40 Gm, globulin 33 Gm per 100 ml)

Hemolysis continued at an extremely rapid rate with only minor fluctuations, and blood transfusions were only of transient benefit Hemoglobinuria was generally absent, but was observed on several occasions after transfusions Data obtained by the differential agglutination technic (Dr J F Loutit) confirmed that the transfused blood was very rapidly eliminated The patient died in April 1947

## NATURE OF THE SERUM HEMOLYSIN

Samples of the patient's blood were investigated on several occasions between January and March 1947\* It was repeatedly found that normal group O erythrocytes and the patient's own corpuscles underwent hemolysis in vitro in the patient's serum The hemolytic antibody seemed to be distinct from the cold hemagglutinin antibody and was absorbed on to corpuscles better at 37 C than at lower temperatures The amount of hemolysis was largely determined by the pH of the cor-

\* I am indebted to Dr J F Loutit for blood from this patient and to Dr J F Hawkesley for details of the clinical history

TABLE I  
For Procedures and Remarks see below

Experiment	Serum	Corpuscles	Hemolysis
			%
1a.	Patient*	Patient	Nil
b	Patient*	Normal (O)	5
c	Acidified† patient's serum	Patient	35
d	Acidified† patient's serum	Normal (O)	60
2a.	Inactivated‡ acidified patient's serum	Normal (O)	10
b	Inactivated‡ acidified patient's serum	Normal (O)	6
c	Inactivated‡ acidified patient's serum.	Normal (O)	3
3	Inactivated‡ acidified patient's serum	Normal	(a) 20 (b) 70
4a.	Inactivated‡ acidified patient's serum	Normal	20
b		Normal	2
c	Fresh† acidified patient's serum.	Normal	25
d	Fresh† acidified patient's serum	Normal	50
5a	Inactivated‡ acidified patient's serum	Patient	(1) 10 (2) Nil.
b	Inactivated‡ acidified patient's serum.	Normal	(1) 55 (2) 25
c.	Inactivated‡ acidified patient's serum	Patient	(1) 10 (2) 20
d	Inactivated‡ acidified patient's serum	Normal	(1) 55 (2) Nil

1 a b c and d PROCEDURE The corpuscle serum suspensions were centrifuged after 30 minutes at 37 C. REMARKS Demonstrates the effect of pH on the hemolysis of the patient's corpuscles and of normal group O erythrocytes. The patient's own corpuscles are less sensitive than are the normal erythrocytes.

2. PROCEDURE (a) The corpuscles were sensitized in the patient's serum for 30 minutes at 37 C. The suspension was then centrifuged; the deposited corpuscles were washed in warm saline and resuspended in fresh normal acidified† serum and incubated at 37 C for a further 30 minutes. (b) Same as (a) but the corpuscles were sensitized in the patient's serum at 16 C. (c) Same as (a) but the corpuscles were sensitized in the patient's serum at 2 C. REMARKS Demonstrates that the hemolysin is less readily absorbed at temperatures below 37 C.

3. PROCEDURE The corpuscle serum suspension was incubated for 30 minutes at 37 C, then centrifuged and the corpuscles washed once in warm saline. The sensitized corpuscles were then divided into two equal portions (a) and (b). To (a) was added a volume of heated normal serum at its natural pH (8.0) to (b) was added heated acidified normal serum (pH 7.0). Both tubes were held at 37 C for 30 minutes, then centrifuged and fresh acidified normal serum added to the deposited corpuscles (pH 7.0 approx) and the suspensions incubated at 37 C for a further 30 minutes. REMARKS Shows that the hemolysin is absorbed best at a relatively acid reaction and may be liberated from the corpuscles into serum of a more alkaline reaction (a).

4. PROCEDURE (a) The corpuscle serum suspension was incubated at 37 C for 30 minutes, then centrifuged. To the deposit was added absorbed guinea pig serum. The tube was incubated at 37 C for one hour. (b) Unsensitized normal corpuscles were suspended in absorbed guinea pig serum and incubated at 37 C for one hour (control for a). (c) Incubated at 37 C for one hour. (d) Same as (c) but with the addition of absorbed guinea pig serum. REMARKS (a and b) Sensitized normal corpuscles are hemolyzed by fresh guinea pig serum complement. (c and d) Hemolysis is increased in the presence of additional guinea pig serum complement.

5. PROCEDURE (a) (1) The suspension was incubated at 37 C for 30 minutes, then centrifuged. Acidified fresh normal serum was added to the deposit and the tube incubated at 37 C for one hour.

puscle-serum suspension, hemolysis was maximal at about pH 6.8 to 7.0 and was inhibited below pH 6 and above pH 8, and there was but a trace of hemolysis in unacidified serum. This restricted pH-hemolysis range seemed due to the hemolysin being poorly absorbed at the alkaline side of neutrality, and it was demonstrated that hemolysin absorbed at the optimum pH was liberated again if the sensitized corpuscles were suspended in a more alkaline serum. The antibody was found to be thermostable and withstood heating to 56°C for thirty minutes. Complement was required for hemolysis and either fresh human serum or guinea pig serum was satisfactory. The titer of the hemolysin (determined against normal corpuscles under what was thought to be optimum conditions) was 1:8 (final serum dilution).

Absorption experiments showed that normal corpuscles absorbed hemolysin active against patient's corpuscles and vice versa, and there seemed to be no difference in sensitivity to the hemolysin between the patient's immature corpuscles (reticulocytes) and her mature erythrocytes. Repeatedly, the patient's corpuscles were shown to be less sensitive to hemolysis than were normal erythrocytes.

Some of the data on which the above description is based are recorded in table 1.

### DISCUSSION

Although the cause was obscure there can be little doubt as to the nature of the disorder from which the subject of this report was suffering, the negative family history, the severe anemia and high reticulocytosis, the presence of cold hemagglutinins, the positive Coombs test, the relapse after splenectomy and the transient benefit of blood transfusions due to a rapid elimination of the transfused corpuscles, and the presence of an abnormal auto- and isohemolysin in the serum all indicate a severe idiopathic acquired hemolytic anemia.

The presence in the patient's serum of an abnormal hemolysin is the most unusual feature and has seldom been observed. It is probably only in the most severe forms of hemolytic anemia when autoantibodies are being formed in large

(2) Further patient's corpuscles were added to supernatant. The suspension was centrifuged after 30 minutes. Fresh acidified normal serum was added to the deposited corpuscles and the tube incubated at 37°C for one hour. (b) Same as (a) except that normal corpuscles were used throughout. (c) Same as (a), except that normal corpuscles were used in the second stage of the experiment to test for the absorption of the hemolysin by the patient's corpuscles. (d) Same as (a) except that patient's corpuscles were used in the second stage of the experiment to test for the absorption of the hemolysin by the normal corpuscles. **REMARKS** Demonstrates the cross absorption of hemolysin between patient's and normal corpuscles and the relative insensitivity of patient's corpuscles compared with the normal.

\* Serum not acidified (pH approximately 8.0)

† Serum acidified by the addition of 10 per cent by volume of N/4 HCl. The pH after the addition of the corpuscles and incubation at 37°C for 30 minutes was approximately 7.0.

‡ Serum inactivated by heating to 56°C for 30 minutes, acidified with 10 per cent by volume of N/4 HCl after inactivation.

Fresh guinea pig serum was absorbed with equal volumes of washed normal human corpuscles for 30 minutes at 2°C. The serum was used at a final dilution of 1 in 5.



amounts that there is sufficient for detention in the serum over and above that absorbed on to the patient's own corpuscles, this probability, and the fact that adjustment of pH to an optimum for hemolysis is important in the demonstration of hemolysins of the type now described, perhaps accounts for the fact that observations similar to the present have seldom been reported.

In France, however, about forty years ago, the role of hemolysins in acute hemolytic anemia was well recognized (Chauffard and Troisier,<sup>9</sup> Chauffard and Vincent<sup>10</sup>), and these early papers and some others are referred to by Dameshek

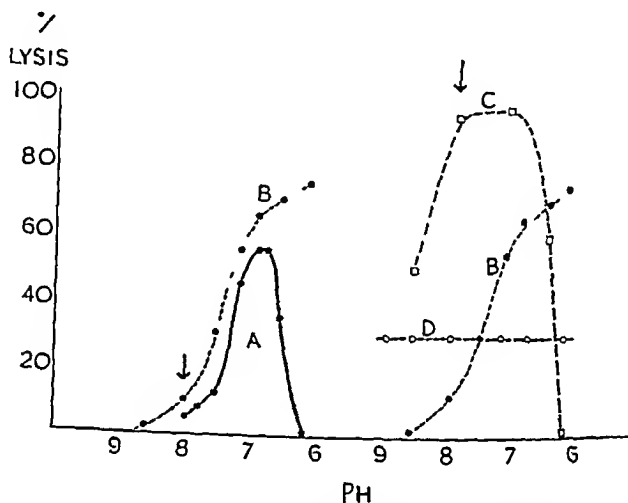


FIG. 1.—On the left A (continuous line) a pH hemolysis curve for the hemolysis of normal corpuscles by the patient's (Miss B's) serum and B (interrupted line) the effect of pH on the absorption of the hemolysin; there was only a trace of hemolysis in unacidified serum indicated by the black arrow due to absorption being inhibited by increasing alkalinity.

On the right C (interrupted line) the effect of pH on the absorption of a cold hemolysin present in the serum of a patient suffering from cold hemoglobinuria, and D (interrupted line) the absence of any effect of pH on the absorption of anti A (or anti B) isohemolysins. Curve B is reproduced for comparison.

and Schwartz<sup>11</sup> in their review of acute hemolytic anemia. Following these early papers, however, the association of hemolysins and acute hemolytic anemia seems to have been forgotten until in 1938 Dameshek and Schwartz<sup>12</sup> published 3 cases of their own. More recent reports are those of Farrar, Burnett and Steigman,<sup>13</sup> David and Minor,<sup>8</sup> Neber and Dameshek,<sup>14</sup> and of Ellis, Wollerman and Stetson.<sup>15</sup> Only in the report of David and Minor has the effect of pH on the demonstration of hemolytic activity been investigated. These authors observed a substantial increase in hemolysis when the corpuscles were suspended in serum acidified with 5 per cent N/3 HCl instead of in unacidified serum, in one instance an increase from 47 to 111 mg in the concentration of liberated hemoglobin.

As has already been mentioned, the effects of pH on the action of guinea pig

and human serum complement are well recognized. In the present instance, there was evidence that the absorption of antibody was also controlled by pH, and that it was this effect which was responsible for the comparatively restricted pH range between which hemolysis could be demonstrated.

It was of interest to contrast the behavior of this patient's hemolysin with two other types of antibody, the anti-A and anti-B isohemolysins and a cold hemolysin from a patient suffering from cold hemoglobinuria. The effect of pH on the absorption of these three types of hemolytic antibodies is indicated in figure 1. The left hand curve (A) represents the pH range within which hemolysis of normal corpuscles by Miss B's serum could be demonstrated, the range was

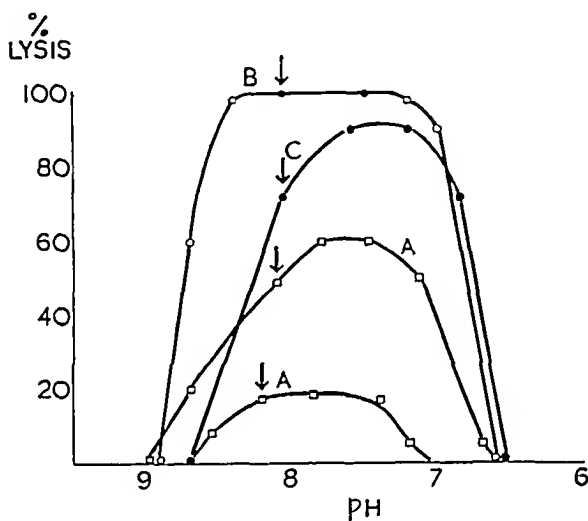


FIG. 2.—The effects of pH on the hemolysis of group A corpuscles by the anti A isohemolysin (curves A) and of group B corpuscles by the anti B isohemolysin (curve B). Curve C indicates the effect of pH on the hemolysis of group O corpuscles by a cold hemolysin present in the serum of a patient suffering from cold hemoglobinuria. The black arrows indicate the amount of hemolysis produced by unacidified serum.

approximately pH 6 to pH 8 with an optimum about pH 7. Curve B represents the effect of pH on the absorption of the hemolysin, i.e., the amount of hemolysis observed when corpuscles sensitized in inactivated serum at a range of pH between 6 and 9 were subsequently resuspended and incubated in fresh normal serum at pH 7.\* The absorption of antibody diminished with increasing alkalinity, and

\* Washed normal erythrocytes were suspended at a final concentration of 5 per cent in volumes of patient's inactivated serum whose pH had been adjusted from 9 to 6 by the addition of 10 per cent by volume of HCl ranging in strength from N/20 to N/2.5 and NaOH ranging in strength from N/20 to N/5. The corpuscle-serum suspensions were centrifuged after thirty minutes at 37°C and the deposited corpuscles washed once in saline warmed to 37°C. Finally volumes of fresh normal serum at pH 7.0 were added to the deposited corpuscles and the tubes incubated at 37°C for 30 minutes. The amount of hemolysis in each tube was dependent upon the amount of hemolysin absorbed in the first stage of the experiment.

it is this fact that probably reduced to a mere trace the amount of hemolysis caused by unacidified serum. In the right hand diagram in figure 1, curve B is reproduced again. Curve C represents the effect of pH on the absorption of the cold hemolysin and the line D shows that pH has no effect on the absorption of anti A (or B) isohemolysin. In figure 2 are shown as a contrast to curve A of figure 1, pH hemolysis curves (the summation of effects of pH on the absorption of the antibody and upon the action of human serum complement) for the hemolysis of normal corpuscles by anti-A and anti-B isohemolysins (curves A and B) and by the cold hemolysin (C). The black arrows indicate the amount of hemolysis produced by unacidified serum at approximately pH 8, and show that this is almost maximal. In the case of curves A and B (fig. 2), the relatively wide range of pH within which hemolysis will take place is due to pH affecting the activity of serum complement alone and not the absorption of the antibody. The range for the cold hemolysin (curve C) is slightly more restricted on the alkaline side, in this case, there is some impairment of absorption of antibody between pH 8 and 9. It is noteworthy that the pH range for the action of Miss B's hemolysin quite closely corresponds to the pH range within which the erythrocytes from patients with nocturnal hemoglobinuria will undergo hemolysis in normal serum (Dacie and Richardson<sup>4</sup>).

It is remarkable that the effect of pH on the activity of the three different types of hemolysins described in this paper was different in each case. Such differences no doubt reflect subtle differences in the composition of the protein complexes concerned. From the practical point of demonstrating the hemolytic nature of these antibodies *in vitro*, the effect of pH cannot altogether be disregarded.

#### SUMMARY

The presence is recorded of an abnormal hemolysin in the serum of a patient with severe acquired hemolytic anemia. Its activity *in vitro* was determined by the pH of the corpuscle-serum suspension, the optimum pH was about 6.8 to 7.0 and there was inhibition above pH 8 and below pH 6. This pH range is contrasted with that of other human serum hemolytic systems, it is similar to that found in nocturnal hemoglobinuria.

#### REFERENCES

1. OSBORN J. W. B. Influence of pH on the action of hemolytic complement. *Biochem J.* 28: 413, 1934.
2. SEIFTER S., PILLEMER L. AND ECKER E. E. Immunochemical studies on human serum. 2. *In vitro* studies on the stability of human complement and its components. *J. Immunol.* 47: 195, 1943.
3. HAM J. H. Studies on destruction of red blood cells. I. Chronic hemolytic anemia with paroxysmal nocturnal hemoglobinuria: an investigation of the mechanism of hemolysis, with observations of five cases. *Arch. Int. Med.* 64: 1271, 1939.
4. DACIE J. V. AND RICHARDSON N. The influence of pH on hemolysis *in vitro* in nocturnal hemoglobinuria. *J. Path. & Bact.* 55: 375, 1943.
5. VAN DEN BERGH A. A. H. Ueber die Hämolyse bei der paroxysmalen Hämoglobinurie. *Berl. klin. Wschr.* 46: 1609, 1909.
6. HANNEMA L. S. AND RYTMA J. R. Investigations into a case of paroxysmal hemoglobinuria. *Lancet* 2: 1217, 1922.

- <sup>7</sup> WAGLEY, P F ZINKHAM, W H , AND SIEBEN, A A A note on studies of hemolysis in paroxysmal (cold) hemoglobinuria *Am J Med* 2 342, 1947
- <sup>8</sup> DAVID J K. AND MINOT, A S Acute hemolytic anemia in infancy, Case with demonstration of hemolytic activity of serum *Am J Dis Child* 68 327, 1944
- <sup>9</sup> CHAUFFARD A , AND TROSIER, J Anémie grave avec hémolysine dans le serum ictère hémolysinique *Sem méd Paris* 28 345 1908
- <sup>10</sup> — AND VINCENT C Hémoglobininurie hémolysinique avec ictère polycholique aigu *Sem méd Paris* 29 601 1909
- <sup>11</sup> DAMESHEK, W , AND SCHWARTZ, S O Acute hemolytic anemia *Medicine* 19 231, 1940
- <sup>12</sup> —, AND — The presence of hemolysins in acute hemolytic anemia *New England J Med* 218 75 1938
- <sup>13</sup> FARRAR, G F BURNETT, W E , AND STEIGMAN, A J Hemolytic anemia and hepatic degeneration cured by splenectomy *Am J M Sc.* 200 164 1940
- <sup>14</sup> NEBER J , AND DAMESHEK W The improved demonstration of circulating antibodies in hemolytic anemia by the use of bovine albumin medium *Blood* 2 371 1947
- <sup>15</sup> ELLIS L B WOLLERMAN, O J , AND STETSON R P Autohemagglutinins and hemolysins with hemoglobinuria and acute hemolytic anemia, in an illness resembling infectious mononucleosis *Blood* 3 419 1948

# OBSERVATIONS ON THE INFLUENCE OF THE HYPOPHYSIS AND THE ADRENAL CORTEX ON BLOOD PLATELET LEVELS

By ELIJAH ADAMS, M D \*

THE MAMMALIAN blood platelet remains the formed blood element about which there is little significant information. Major areas of ignorance include the factors concerned with the regulation and the mechanism of platelet formation, release, and utilization, as well as with the exact role of platelets in vascular hemostasis and plasma coagulation.<sup>2</sup> The observations described in this paper were planned to examine the hypothesis that the circulating level of blood platelets might be subject to the influence of certain endocrine secretions. Although no clear indication for such a relationship exists, several lines of evidence are consistent with this working hypothesis. First, a variety of stressful stimuli, including fever,<sup>11</sup> severe exercise,<sup>15</sup> anoxia,<sup>16</sup> hemorrhage,<sup>21</sup> trauma<sup>28</sup> and surgery<sup>1</sup> are reported to result in significant elevation of the platelet count. Since such conditions have as one common factor the stimulation of the pituitary-adrenal cortex system,<sup>17-24</sup> it seemed reasonable to evaluate the possibility that these glands exercise a direct influence on the mechanisms determining the level of circulating platelets.

Secondly, much recent work has revealed a relationship between the activity of several endocrine glands and processes of hematopoiesis involving both the red and white cell series.<sup>13</sup> The anemia which follows hypophysectomy<sup>7-19-27</sup> and the control of lymphocytes exerted by the pituitary-adrenal cortex system<sup>1</sup> represent the more clearly established correlations between endocrine secretions and processes concerned with hematopoiesis.

Finally, there is some evidence that hemostatic vascular reactions, believed to involve the blood platelets,<sup>3-28-30</sup> may be altered by endocrine influences. Ungar<sup>29</sup> presented data indicating that the spleen, activated by the pituitary and the adrenal cortex, secretes a substance effective in shortening bleeding time and increasing capillary resistance.

Observations directed specifically toward a possible endocrine influence upon platelet levels are few in number. Estrogens, administered in massive doses over a period of weeks, have been reported to reduce the platelet counts of dogs and monkeys to purpuric levels,<sup>6-8</sup> the final picture being that of an aplastic anemia in which all the cellular components, both of the peripheral blood and bone marrow, were at low levels. Shekiet and associates<sup>22</sup> observed an average increase of 76 per cent in the blood platelets during the terminal postoperative week in adrenalectomized rats. A report by Dalton, Masson and Selye<sup>9</sup> describes a similar steady rise in platelets following bilateral adrenalectomy in rats. The results of sham

From the Department of Physiological Chemistry Yale University New Haven Conn

Supported by grants from the James Hudson Brown Memorial Fund and from the U S Public Health Service

\* National Institute of Health Senior Research Fellow present address Department of Medicine New Haven Hospital New Haven Conn

operation are mentioned in neither report, however, and the well-substantiated phenomenon of a platelet elevation following all types of surgery<sup>1</sup> makes it impossible to ascribe specifically to the absence of the adrenals the platelet changes reported in these two studies. Zondek and Kaatz<sup>2</sup> reported a moderate reduction in the platelets of men one hour following the administration of small doses of adrenal cortex extract (Richter—cortigen), and an increase following oral thyroid and parenteral thyroxin or thyrotrophic hormone. Following castration of male and female albino rats a significant drop in platelets lasting several months has been described, as well as a gradual elevation of platelets following the subcutaneous injection of gonadal extract.<sup>4</sup>

### MATERIALS AND METHODS

The animals used in this study were male Sprague Dawley rats weighing between 175 and 300 Gm., male and female mice of both the A and the CBA strain between 8 and 12 weeks of age and young adult male rabbits of mixed strain. All animals were kept in air-conditioned rooms at controlled temperatures and fed a standard diet composed of Purina Lab Chow (rats and mice) or Purina Rabbit Chow.

The platelet counting method employed is described in detail elsewhere.<sup>2</sup> In brief the method for rats and mice was as follows: By heart puncture a small standard quantity of blood (0.1–0.2 cc.) was aspirated into a 2-cc. syringe containing a measured volume of sodium oxalate solution. A quantitative dilution of the blood having been made in the original syringe the contents were mixed, transferred to a test tube and allowed to remain undisturbed until a clear layer of diluted plasma appeared as a result of the sedimentation of erythrocytes and leukocytes. This layer was then sampled with a capillary pipet and the platelets counted in a hemocytometer of conventional type. The method for rabbits was identical in essentials except that blood was drawn from the ear artery rather than from the heart. In the case of mice it was necessary to sacrifice an animal for each determination, the heart puncture being performed after opening the chest. Serial counts could be easily made both in rats and rabbits.

Splenectomy and bilateral adrenalectomy were performed on rats and mice in the usual manner under ether anesthesia using a clean but not sterile technic. Following bilateral adrenalectomy mice were given routinely a single subcutaneous injection of desoxycorticosterone acetate in sesame oil (0.25 cc. containing 1.25 mg.), both rats and mice were given a 1 per cent solution of NaCl as drinking water following adrenalectomy. In many but not all cases completeness of adrenalectomy was checked by autopsy.

Male, Sprague Dawley rats, hypophysectomized at about 2 months of age, were obtained from the Hormone Assay Laboratory, Inc., Chicago. Hypophysectomy was considered complete if the animals failed to gain weight and if a marked degree of testicular atrophy appeared.

Aqueous adrenal cortex extract (Wilson) was the preparation of cortical hormone used and was administered to rats subcutaneously in doses of 1 cc. per 100 gm. body weight. Injected control fluids such as physiologic saline and water were given in the same doses. Mice received 0.25 cc. of aqueous adrenal cortex extract subcutaneously; rabbits were given 10 cc. of this preparation subcutaneously.

### RESULTS

#### *Adrenal Cortex Extract in the Intact Animal*

Large single doses of aqueous adrenal cortex extract were found to be without influence on the platelet counts of mice, rats and rabbits. Mice (CBA strain) were sacrificed in groups of 4 to 10 individuals at intervals from fifteen minutes to forty-eight hours following hormone. A group of 16 rats was subjected to platelet counts immediately before, three and twenty-four hours after the injection of adrenal cortex extract. Six rabbits were followed with serial platelet counts at intervals of 1, 4, 8, and 24 hours after hormone. At no interval following injection in any

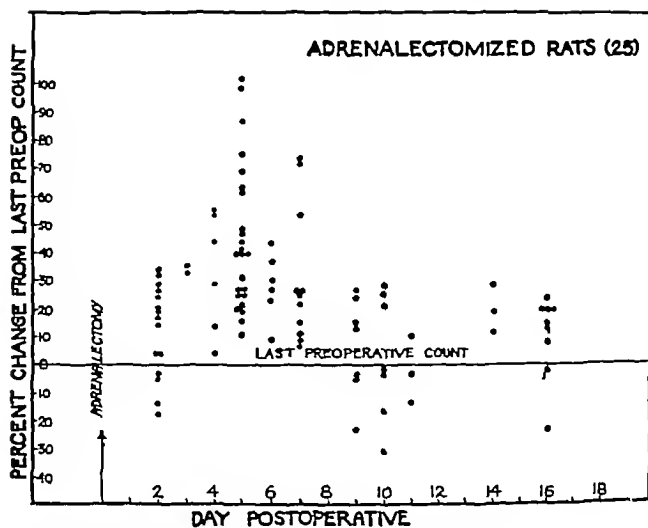


FIG. 1.—Serial changes in the platelet count following adrenalectomy each point represents the percentage change from the last preoperative count in a given rat.

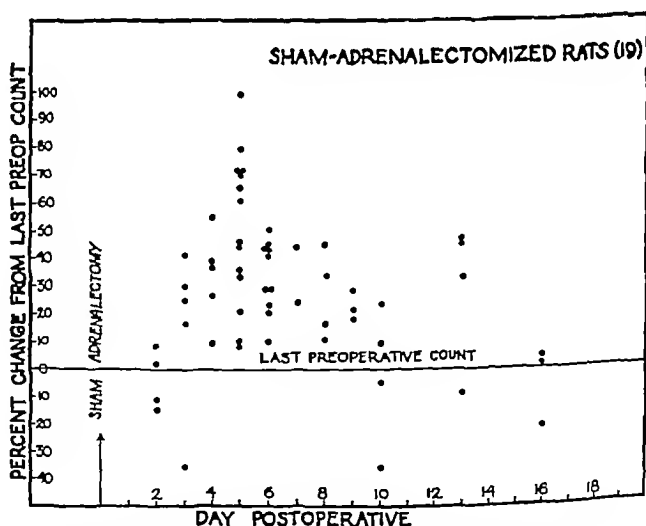


FIG. 2.—Serial changes in the platelet count following sham adrenalectomy each point has same significance as in figure 1. Note similarity to the response after adrenalectomy in figure 1.

of these species, could consistent, significant differences from the control values be detected

*Adrenalectomy*

Platelet counts were performed serially following adrenalectomy in a group of 25 male Sprague-Dawley rats, and following sham-adrenalectomy in a group of 19 similar animals. Both types of operation were carried out in identical fashion, except that in the control operation a piece of perirenal fat was removed from the vicinity of each kidney without disturbing the adrenal gland. The similarity of the platelet response, both in magnitude and duration, can be seen from inspection of figures 1 and 2. In these figures, each point represents the calculated percentage difference between the platelet count on a particular postoperative day in a given animal, and the preoperative count in the same individual. Additional data, covering only the first five postoperative days, revealed no significant difference in the platelet rise following adrenalectomy and sham-adrenalectomy in mice of the A strain.

*Adrenal Cortex Extract Following Adrenalectomy*

In contrast to the failure of adrenal cortex extract to influence the platelet level of intact rats, comparable doses of hormone given to rats following adrenalectomy were found to produce a consistent reduction in the platelet count as detected by comparing counts made in the same animal immediately before and three hours after administration. Individual values for the per cent reduction in platelet numbers ranged between 9 per cent and 28 per cent, the consistency of the direction of change being more striking than its magnitude. Rats were first examined for platelet changes following hormone administration four or five days after adrenalectomy, and the same rats, in most instances, were used for repeat observations at longer periods—between ten and sixty days—postoperatively. A control group of rats subjected to sham-adrenalectomy and examined five to eleven days following operation, was found to show no consistent platelet response to adrenal cortex extract. It should be mentioned that the group of rats originally used to examine the effects of adrenal cortex extract on platelets in the intact animal was subsequently subjected to adrenalectomy or sham-adrenalectomy for the postoperative trial of hormone, so that with a few exceptions, the same group of individual rats served both as control and experimental animals.

In some, but not all, instances, autopsy was performed on rats allowed to survive for long periods (up to sixty days) after adrenalectomy. In a few of these animals regenerated adrenal tissue was found, and the data discarded. No animal was used in the adrenalectomy series unless removal of both adrenals intact had been accomplished. It was considered that adrenalectomy was functionally complete at the five-day interval, even though adrenal regeneration might have occurred many days subsequently. No adrenal tissue was found in any of a group of 13 rats autopsied five to seven days following adrenalectomy.

In an attempt to control these observations further, comparable doses of physiologic saline were given subcutaneously to a group of adrenalectomized rats on the fifth postoperative day. The unexpected finding was made that in the adrenalectomized, but not the sham-operated rat, this treatment too was followed by a



fall in platelets comparable in magnitude to that observed after administration of adrenal cortex extract. In contrast, distilled water given in similar quantity to a group of adrenalectomized rats was succeeded by no significant change in the platelet count. These data are summarized in table 1.

The unlikely possibility that a reduction in the platelet count of such magnitude might be ascribed to hemodilution in the adrenalectomized rat, brought about by the actual volume of adrenal cortex extract or saline injected, was tested by following the change in hemoglobin\* concentration three hours after the subcutaneous administration of physiologic saline to a small number of rats five days after adrenalectomy or sham-adrenalectomy. Under these conditions, the maxi-

TABLE 1—Comparison of average percent change in the platelet count immediately before and 3 hours after the administration of several different preparations to intact, adrenalectomized and sham-adrenalectomized rats

Operative Group	Material Injected	Number of Animals	Days Postoperative	Percent Change in Platelets 3 Hours After Injection
Intact	Adrenal Cortex Extract	16		+4 ± 2.6
Sham Adrenalectomy	Adrenal Cortex Extract	10	5-11	0 ± 2.1
Adrenalectomy†	Adrenal Cortex Extract	16	4-5	-17 ± 2.3
Adrenalectomy†	Adrenal Cortex Extract	12	10-60	-11 ± 3.2
Sham Adrenalectomy	Saline	10	5-7-13	-2 ± 2.2
Adrenalectomy	Saline	7	5-7	-18 ± 3.0
Adrenalectomy	Water	6	5	+1 ± 1.6

\* Means and standard errors

† Values significantly ( $p < 0.01$  by  $t$  test) lower than all other mean values shown. No significant differences between any other two sets of means.

imum reduction in hemoglobin was 7 per cent below the preinjection level, the mean for five animals being a fall of 4 per cent.

### Hypophysectomy

Male Sprague-Dawley rats hypophysectomized at about 2 months of age were followed with serial platelet counts. All counts made within a three week period after hypophysectomy were not included in analyzing the data, because of possible nonspecific effects on the platelet level of the operation itself. As can be seen from table 2, the average value for a group of platelet counts in 23 hypophysectomized rats was significantly, although not strikingly, lower than the average for a group of 71 intact rats of the same sex, strain and approximate age.

\* Hemoglobin was determined in the Coleman Jr Spectrophotometer by the alkaline hematin method.<sup>22</sup>

The phenomenon of a reduction in platelet numbers following the administration of adrenal cortex extract to adrenalectomized rats might suggest the possibility of a similar change in hypophysectomized rats after a postoperative interval sufficient to permit adrenal atrophy. No significant difference was noted, however, between the preinjection and three-hour postinjection platelet counts of a group of 6 rats given subcutaneous adrenal cortex extract twenty-eight days after hypophysectomy.

TABLE 2.—Average platelet values in intact and hypophysectomized rats

	Number of Animals	Number of Counts	Platelets/cmm blood (X 1000)
Intact	71	188	989 $\pm$ 14.8
Hypophysectomized	23	47	854 $\pm$ 20.4†

\* Means and standard errors

† Significantly lower than control mean ( $p < 0.01$  by  $t$  test)

#### *Platelet Response to Splenectomy in Intact and Hypophysectomized Rats*

Of all types of surgery, splenectomy is generally followed by the largest and most enduring postoperative elevations in the platelet count.<sup>25</sup> There is some evidence<sup>8</sup> that this phenomenon is due to removal of the large complement of reticulo-endothelial cells in the spleen, which may normally play a role in clearing platelets from the circulation. An alternative explanation holds that the spleen normally exerts an inhibitory effect on platelet formation in the bone marrow, an activity quite clearly demonstrated by Dameshek and Miller<sup>10</sup> in patients with essential thrombocytopenic purpura.

In contrast to the minimal reduction of the platelet count as a result of hypophysectomy, the platelet response following splenectomy was found to be markedly depressed in the hypophysectomized rat as compared with the effects of splenectomy in the intact rat.\* On the fifth and sixth day postsplenectomy in the otherwise intact rat, maximum platelet levels were observed, representing percentage increases roughly 100 per cent above the preoperative level. At a similar interval after splenectomy in hypophysectomized rats, increases averaging about 40 per cent were noted. These data are expressed in figures 3 and 4, in which each point plotted represents the calculated percentage difference between the platelet count in a given rat at the indicated postoperative level and the preoperative count in the same animal.

A small number of intact and hypophysectomized rats, before and five days after splenectomy, were autopsied to provide marrow specimens examined by the method described by Mayer and Ruzicka.<sup>18</sup> This technic permits the microscopic examination of a longitudinal section of the entire femoral bone marrow fixed in

\* Most of the hypophysectomized rats were subjected to splenectomy at least 1 month following hypophysectomy. In 4 rats, splenectomy was performed only eight days following hypophysectomy; no difference in platelet response was observed in this group as compared with the results of splenectomy in the remaining hypophysectomized animals.

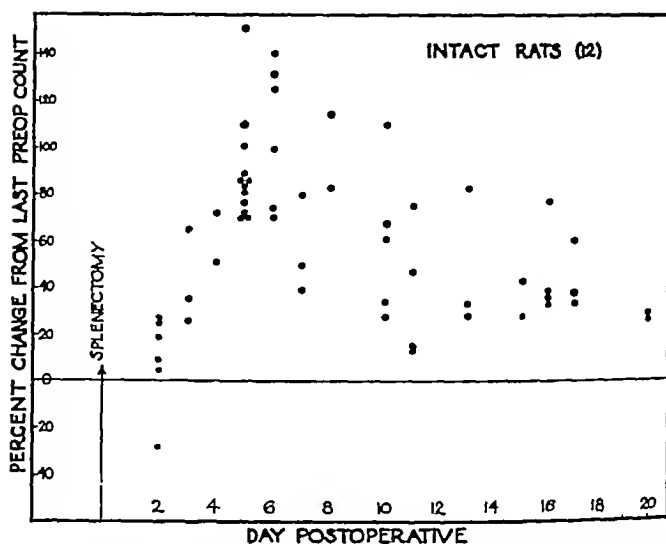


FIG 3 —Platelet response to splenectomy in the intact rat each point represents the percentage change from the last preoperative count in a given rat

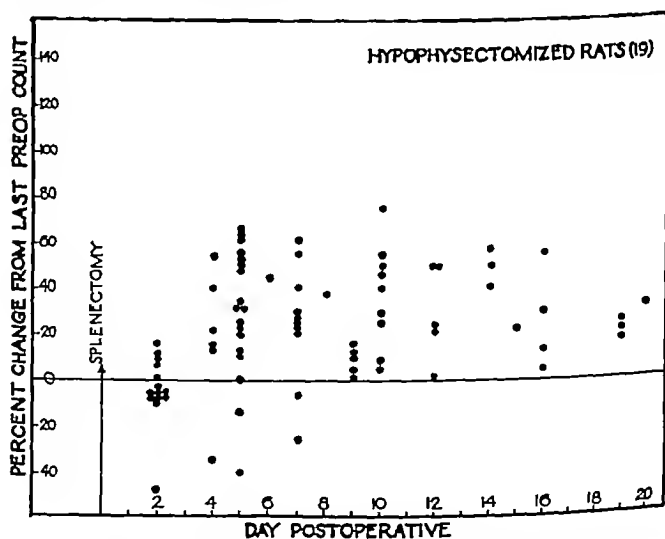


FIG 4 —Platelet response to splenectomy in previously hypophysectomized rats Each point has same significance as in figure 3

situ, and was thought to be the best method available for enumerating bone marrow elements of infrequent occurrence such as megakaryocytes. The megakaryocytes seen in 20 high-dry fields (magnification 400 diameters) of each bone mar

row section were counted, the results, recorded in table 3, indicate a significant reduction of megakaryocytes in the hypophysectomized rat both before and after splenectomy, but no significant change in the number of megakaryocytes following splenectomy either in intact or hypophysectomized rats. The failure to note quantitative changes in megakaryocytes following splenectomy agrees with the observations of Higgins and Stasney<sup>14</sup> that marrow imprints made following splenectomy in otherwise intact rats revealed no significant increase in the number of megakaryocytes, despite a large postoperative rise in circulating platelets.

Inspection of megakaryocytes in Giemsa-stained smears of the bone marrow of rats in the four categories cited above—intact and hypophysectomized, before and after splenectomy—revealed no qualitative morphologic differences in megakaryocytes, such as depression of platelet formation, absence of granularity, and other changes of the type described by Dameshek and Miller<sup>10</sup> in idiopathic thrombocytopenic purpura of man.

TABLE 3—Bone marrow megakaryocytes in hypophysectomized and intact rats before and 5 days after splenectomy

	Relation to Splenectomy	Number of Animals	Number of Megakaryocytes*
Intact	Before	6	145 $\pm$ 10.7
	After	3	142 $\pm$ 6.4
Hypophysectomized†	Before	3	98 $\pm$ 9.4
	After	3	75 $\pm$ 4.7

\* Number per 20 high dry fields. Means and standard errors.

† Both hypophysectomized means significantly lower than either intact mean ( $p < 0.01$  by  $t$  test). No significant difference before or after splenectomy in either group.

Other relevant observations included changes in the weight and histologic appearance of spleens removed from hypophysectomized rats. As reported earlier by Perla<sup>20</sup> hypophysectomy is followed by a progressive reduction in spleen weight. In the present study, the spleen/body-weight ratio in rats a month after hypophysectomy was found significantly lower than the same value in intact rats of corresponding age. Histologic changes in the spleens of hypophysectomized rats included the presence of fewer megakaryocytes in this tissue as well as in the bone marrow, and a reduction of mitotic activity in the germinal centers of lymphoid follicles. A fuller account of the morphologic alterations in the spleen after hypophysectomy can be found in Perla's paper,<sup>20</sup> which also describes hyperplasia of the germinal centers of splenic lymph follicles, and an increased number of megakaryocytes, both in spleen and bone marrow, in rats given extracts of dried beef pituitary.

## DISCUSSION

The results described do not support the hypothesis that blood platelets are influenced in any specific or significant way by the hormones of the pituitary or adrenal cortex. The almost exact similarity in response of platelets to sham opera-

tion and to adrenalectomy would seem to confirm the suspicion that those reports describing large increases in platelets after adrenalectomy, were simply observations of the well-known phenomenon of postoperative thrombocytosis seen after major surgery of almost any nature

Of more positive interest, although difficult to relate to other findings, is the observation that the administration either of adrenal cortex extract or physiologic saline is followed by a significant fall in platelets in the adrenalectomized but not in the sham-operated rat. This finding, coupled with the failure of distilled water to influence the platelets of adrenalectomized rats, suggests a possible electrolyte effect, although no further light can be thrown on this question with the data of the present study. Whatever mechanism underlies this observation, the fact that it does not occur in rats some weeks after hypophysectomy suggests that it requires the absence, rather than a moderate relative functional insufficiency, of the adrenals.

Observations of platelet levels following hypophysectomy, and the platelet response to splenectomy of the hypophysectomized rat, raise some interesting questions as to the equilibrium of production and removal rates which must govern the level of circulating platelets. First, the small, if significant, decline in platelets in the hypophysectomized rat, does not by itself suggest any primary action of the pituitary on platelet levels. Changes of this magnitude might be considered part of the picture of generalized tissue atrophy and lowered tissue metabolism following hypophysectomy, just as the reduction of megakaryocytes in the marrow is part of the picture of generalized marrow hypoplasia.

The marked reduction in the thrombocytosis following splenectomy in the hypophysectomized animal, however, suggests an additional possibility. The atrophy of the spleen which occurs after hypophysectomy may quite possibly indicate a reduced functional capacity of this organ, and perhaps other reticulo-endothelial tissue, to remove platelets from the circulation. With such a reduction in level of both platelet-forming and platelet-removing potential, a new equilibrium in the level of circulating platelets might be established, which would not differ greatly from the level in the intact animal. Sudden removal of a large component of reticulo-endothelium, as by splenectomy, might then temporarily unmask the reduced production capacity (by eliminating the balancing factor of platelet removal), and permit its detection in terms of a much depressed platelet response to splenectomy. Such an explanation is of course not uniquely determined by the observed facts, but merely fits them with reasonable simplicity.

#### SUMMARY

Observations were made to investigate possible endocrine influences on blood platelets. Adrenal cortex extract failed to influence the platelet counts of mice, rats, or rabbits. Adrenalectomy and sham-adrenalectomy were followed by almost identical platelet increases in mice and rats. Administration of adrenal cortex extract, or physiologic saline, to adrenalectomized rats was followed by a consistent fall in platelets not observed in sham-adrenalectomized rats, or after administering distilled water to adrenalectomized rats. Platelet levels in hypophysectomized rats were significantly lower than in unoperated controls. Splenec-

tomy in hypophysectomized rats was followed by a maximum rise in platelets markedly lower than following splenectomy in intact rats. Bone-marrow megakaryocytes in hypophysectomized rats were significantly fewer than in intact rats. No changes in megakaryocyte number or morphology appeared following splenectomy either in intact or hypophysectomized rats.

## ACKNOWLEDGMENT

Grateful acknowledgment is made of the frequent advice and aid of Dr. Abraham White.

## REFERENCES

- <sup>1</sup> ADAMS E. Postoperative thrombocytosis. *Arch. Int. Med.* 73: 319, 1944.
- <sup>2</sup> ———. A method for counting blood platelets in small animals. *Yale J. Biol. & Med.* In press.
- <sup>3</sup> AGGELER, P. M., HOWARD, J. AND LUCIA, S. P. Platelet counts and platelet function. *Blood* 1: 472, 1946.
- <sup>4</sup> BANKOW, G. Einfluss der Sexuellen Hormone auf der Blutplättchen. *Beitr. z. path. Anat. u. z. allg. Path.* 88: 113, 1931.
- <sup>5</sup> BEDSON, S. F. Role of the reticulo-endothelial system in the regulation of the number of circulating platelets. *Brit. J. Exp. Path.* 7: 317, 1926.
- <sup>6</sup> CASTRODALE, D., BIERBAUM, O., HEILWIG, E. B. AND MACBRYDE, C. M. Comparative studies of effects of estradiol and stilbestrol upon blood, liver and bone marrow. *Endocrinology* 29: 363, 1941.
- <sup>7</sup> CRAFTS, R. C. Effects of hypophysectomy, thyroidectomy and adrenalectomy on the blood of the adult female rat. *Endocrinology* 29: 596, 1941.
- <sup>8</sup> ———. Effect of estrogens in dog and monkey. *Endocrinology* 29: 606, 1941.
- <sup>9</sup> DALTON, A. J., MASSON, G. AND SELYE, H. Coagulation et éléments figurés du sang pendant le syndrome général d'adaptation. *Bull. et Mémoires de la Soc. Roumaine d'Endocrinologie* 5: 176, 1939.
- <sup>10</sup> DANTESHER, W., AND MILLER, E. B. Megakaryocytes in thrombopenic purpura. *Blood* 1: 27, 1946.
- <sup>11</sup> DEOKWITZ, R. Studien über Blutplättchen. *Folia haemat.* 25: 153, 1920.
- <sup>12</sup> DOUGHERTY, T. F. AND WHITE, A. An evaluation of alterations produced in lymphoid tissue by pituitary-adrenal cortical secretion. *J. Lab. & Clin. Med.* 32: 584, 1947.
- <sup>13</sup> GORDON, A. S. AND CHARLIPPER, H. A. The endocrine system and hemopoiesis. *Ann. N. Y. Acad. Sci.* 48: 615, 1947.
- <sup>14</sup> HIGGINS, G. M., AND STASNEY, J. Changes in marrow and blood following splenectomy in albino rats. *Folia haemat.* 56: 189, 1936.
- <sup>15</sup> ISAACS, K. AND GORDON, B. Effects of exercise on the distribution of corpuscles in the blood stream. *Am. J. Physiol.* 71: 106, 1924.
- <sup>16</sup> KEMP, G. T. Effect of altitude on blood corpuscles and blood plates. *Bull. Johns Hopkins Hosp.* 15: 177, 1904.
- <sup>17</sup> LONG, C. N. H. Conditions associated with the secretion of the adrenal cortex. *Fed. Proc.* 6: 461, 1947.
- <sup>18</sup> MAYER, E. AND RUZICKA, A. Q. Method for studying numerical and topographical problems in whole femoral marrow of rats and guinea pigs. *Anat. Rec.* 93: 213, 1945.
- <sup>19</sup> MEYER, O. O., STEWART, G. E., THEWLIS, E. W. AND RUSCH, H. P. The hypophysis and hemopoiesis. *Folia haemat.* 57: 970, 1937.
- <sup>20</sup> PERLA, D. Relation of hypophysis to spleen. *J. Exper. Med.* 63: 599, 1936.
- <sup>21</sup> RICHARDSON, F. L. Effect of severe hemorrhage on the number of blood plates. *J. Med. Res.* 13: 99, 1904.
- <sup>22</sup> SANFORD, A. H., SHEARD, C. AND OSTERBERG, A. E. The photometer and its use in the clinical laboratory. *Am. J. Clin. Path.* 3: 405, 1933.
- <sup>23</sup> SHECKETT, H. A., FRIEDMAN, D. L. AND NICE, L. B. Thrombocytes and leucocytes in the blood of adrenalectomized rats. *Proc. Soc. Exper. Biol. & Med.* 32: 608, 1935.
- <sup>24</sup> TEFPERMAN, J. AND ENOEL, F. L. *Metabolic Determinants of Adrenal Size and Function*. New York: Josiah Macy, Jr. Foundation, 1942.

- <sup>25</sup> TOCANTINS L. M. Mammalian blood platelet in health and disease *Medicine* 17 155 1938
- <sup>26</sup> UNGAR, G. Endocrine function of the spleen *Endocrinology* 37 329 1945
- <sup>27</sup> VOLLMER E. P. GORDON A. S. LEVENSTEIN I., AND CHARIPPER H. A. Effects of hypophysectomy on blood picture of rat *Endocrinology* 25 970 1939
- <sup>28</sup> WRIGHT H. P. Changes in the number of blood platelets after trauma *J. Obst. & Gynaec. Brit. Emp.* 52. 253 1945
- <sup>29</sup> ZONDEK, H., AND KAATZ. Hormonal regulation of number of blood platelets *Brit. Med. J.* 2. 387, 1936.
- <sup>30</sup> ZUCKER M. B. Platelet agglutination and vasoconstriction as factors in spontaneous hemostasis *Am. J. Physiol.* 148 275 1947

# THE EFFECT OF HUMAN PLASMA TRANSFUSIONS ON THE FECAL UROBILINOGEN EXCRETION IN SICKLE CELL ANEMIA

By EUGENE KAPLAN,\* M D , AND S ROBERT LEWIS, M D

IN 1938, Josephs<sup>1a, b</sup> reported a phenomenon occurring in children with hemolytic anemia fecal urobilinogen excretion was observed to decrease following either blood transfusions, plasma injections or injections of concentrates of human or pig plasma These decreases were of variable degree, occurred within a week of treatment, and lasted up to three weeks Changes in the levels of red blood cells or hemoglobin were slight or absent Infection accompanied by erythroblastosis appeared to interfere with this phenomenon Since the original report, this observation has not been confirmed The present report deals with observations on children with sickle cell anemia in which we have attempted to confirm and study further the presence of an antihemolytic factor in plasma by means of fecal urobilinogen excretion

## METHODS

### *Urobilinogen Determination*

Urobilinogen was determined by the method of Watson<sup>2-4</sup> The total feces for periods of two to four days were collected in cardboard containers tightly covered and stored in a refrigerator The total stool was then thoroughly mixed with water and weighed To a 10 Gm sample was then added 300 cc. distilled water 100 cc ferrous sulfate solution, and 100 cc of 10 per cent sodium hydroxide The resultant mixture was then placed in the dark for at least one hour or until the supernatant solution appeared relatively colorless Occasionally as in the case of specimens with very high urobilinogen content the supernatant fluid appeared distinctly yellow Then 50 cc. of filtrate of the original mixture was added to 25 cc. of 20 per cent ferrous sulfate to which was then added 25 cc of 10 per cent sodium hydroxide This mixture was placed in the dark for one half to one hour, at which time its supernatant fluid appeared relatively colorless Fifty cc of filtrate of the mixture to be used was then removed, placed into a separatory funnel and acidified with 5 cc glacial acetic acid Extraction with 100 cc. of petroleum ether was then performed no less than ten minutes having been allowed for the actual shaking The ether layer was then shaken with Ehrlich's aldehyde reagent followed by shaking with saturated sodium acetate solution The proportion of Ehrlich's reagent to acetate solution was maintained at 1 to 3 This was repeated until no further color developed The total volume of colored solution obtained was measured and the color read in a Klett-Summerson colorimeter using a phenol sulfonphthalein standard Calculation was performed as suggested by Watson Excretion was uniformly expressed as mg urobilinogen per diem average

### *Materials*

The plasma used for therapy was whole plasma For the last 3 transfusions of the 12 administered to Patient I and for all the 4 transfusions administered to Patient V it was obtained from freshly drawn citrated blood of the same blood group as the recipient It was always used within twelve hours All other plasma was derived from Red Cross dried pooled plasma reconstituted just prior to administration

From the Department of Pediatrics New York University College of Medicine and the Children's Medical Service Bellevue Hospital New York City  
\* Rockefeller Foundation Fellow in Pediatrics



TABLE I—Summary of Data during Transfusion Experiments

Pt	Treatment		Collection period	Urobilinogen excretion		Red blood count	Hemoglobin	Reticulocyte	Serum bilirubin	Comment
	Material	Am t	Date	Before	After					
I A M 6 yrs Group B	Plasma I	300 cc	10/20/45	160 mg	23	23 26	70 75			Period I
					36	22	70			
					63					
					82					
					110	30	80			
	Plasma II	30	2/19/46	100		22	65			
					100					
				(100)	52 115					
					50	25	70			
						25 23	70 75		25 28	Period II
	Plasma V	100	9/30/46	330	300 280 340			10		
	Blood VI VII	500 250	12/20/46 12/21/46	250	190 350 140	22	74	14	22 240 250 80	Obstructive jaundice and erythroblas- tosis
	Plasma VIII	350	1/27/47	230 mg	280 185	22 25 25	90 90 80	25	40	Period II

	Plasma IX	300	2/11/47	0-5 6-10 11-15	(185)	360 220 390	1 2 2 8	7 5 9 0	10 35	1 8	Period III
	Plasma X	250	2/28/47	0-5 6-10 11-15	(390)	400 340 540	2 8 3 0 2 4	8 2 9 0 8 5	20 25 20	1 7	
	Plasma XI (Group B)	200	3/12/47	0-5 6-10 11-20 21-25 30-35 5 wks. later 6 wks	(540)	570 400 570 530 590 350 250	3 0 2 4 1 9 2 6 2 7 2 9 2 6	9 0 8 5 8 0 7 5 7 2 7 1 8 0	25 20 25 22 35 30	2 8    1 8	
	Plasma XII (Group B)	200	11/22/47	7 days 2-7	225	458	1 8 2 7	6 5 6 3	30 25	2 2	
	Plasma XIII (Group B)	180	1/12/48	5 days 0-5 6-10	260	370 250	2 0 2 7 2 7	5 6 6 5 8 0	26 22	2 5 2 9 2 3	
II WM 8 yrs	Plasma	100	2/12/46	20 days 0-5 6-10 11-15 20-24	75 mg	38 43 45 65	2 0 2 6	7 5 7 8			



## OBSERVATIONS ON CONTROL SUBJECTS

Urobilinogen determinations were made on a group of 10 control children, either normal or convalescent from some minor illness, and varying in age from 1 year to 12 years. Seven of these patients had two or more determinations. The results conform closely to those reported by Tat, Greenwalt and Dameshek,<sup>3</sup> who used the same technic in infants and children. Urobilinogen excretion varied from 2 mg. to 12 mg. per diem, the amount being roughly proportional to the age and weight of the subject. When expressed as hemolytic index, or mg. urobilinogen excretion for each 100 Gm. estimated total hemoglobin, the resultant values are markedly lower than those observed in normal adults by Miller et al.<sup>4</sup> and also by Watson.<sup>5</sup>

## OBSERVATIONS ON SICKLE CELL ANEMIA SUBJECTS

Urobilinogen excretion was followed in 5 children with sickle cell anemia. The severity and manifestations of the disease were not unusual in these patients who were hospitalized specifically for purposes of this study. The rate of urobilinogen excretion before treatment was at least 5 to 10 times that of the control group, the lowest levels being at least 75 mg. per diem.

## APPARENT CONFIRMATION OF JOSEPH'S HYPOTHESIS

Decreases in urobilinogen excretion were observed in 3 out of 4 cases transfused with whole human plasma, and in 1 infant transfused with the whole blood.

*Case I, A. M.* (Bellevue Hospital #19207-46) a Negro boy aged 10 years was under intensive study for three years. Since the age of 5 he had recurrent attacks of pain in his back and extremities and less frequent mild hemolytic crises. He also had occasional attacks of moderately severe asthmatic bronchitis. The spleen was not enlarged and characteristic roentgenologic changes of chronic hemolytic anemia were present in his skull and phalanges.

Between October 1945 and April 1946 this patient received four plasma transfusions. With the exception of the second one in which only 30 cc. were given and following which stool collections were faulty, each of these was followed by a 50 to 75 per cent decrease in urobilinogen excretion (fig. 1). The effect appeared within five days after treatment and lasted from two to four weeks. There were no significant changes in the levels of hemoglobin, red cells, or serum bilirubin during this entire period.

*Case II, W. M.* (Bellevue Hospital #17381-48) a 10 year old Negro boy was observed for two years. During the previous four years he had been hospitalized elsewhere for annual attacks of abdominal pain, weakness, and icterus and treated with whole blood transfusions. During the present study he remained free of such attacks and had occasional episodes of acute sinusitis. He had moderately severe anemia, icterus and slight splenomegaly.

A single transfusion of human plasma was followed by a decrease in fecal urobilinogen excretion (fig. 4). Pretransfusion excretion was relatively stable. Following the transfusion urobilinogen output decreased 40 per cent and slowly increased to previous levels during the next three weeks.

*Case III, W. D.* (Bellevue Hospital #40130-44) a Negro boy aged 4 years had recurrent attacks of abdominal pain associated with pallor, icterus, and splenomegaly since 1 year of age.

A marked decrease in urobilinogen output followed a single transfusion of plasma. Whereas excretion prior to transfusion fluctuated widely, the decrease afterward was striking, reaching levels of 20 mg. a day, the previous low level was 75 mg. a day. The effect appeared within 5 days and persisted during the next three weeks, terminating suddenly when the patient developed a mild hemolytic crisis.

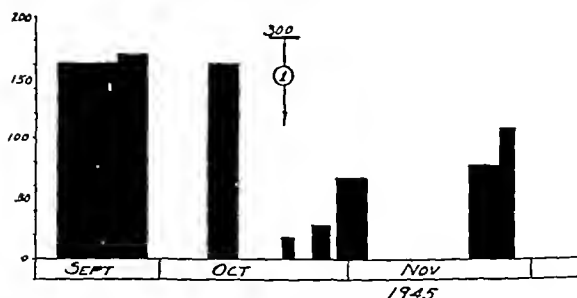


FIG 1.—UROBILINOGEN EXCRETION CASE I A.M. PERIOD I Vertical axis indicates urobilinogen excretion mg per day Note decreased excretion following transfusions

Case IV C C (Bellevue Hospital #26826-47) a Negro female aged 1 year was hospitalized repeatedly since 6 months of age because of recurrent pallor associated with mild respiratory infections and required monthly blood transfusions. Except for pallor icterus and splenomegaly she was an alert well developed and nourished infant.

Urobilinogen excretion decreased 40 per cent following each of two transfusions of citrated whole blood. This effect appeared within a few days after transfusion and gradually disappeared in the next two weeks.

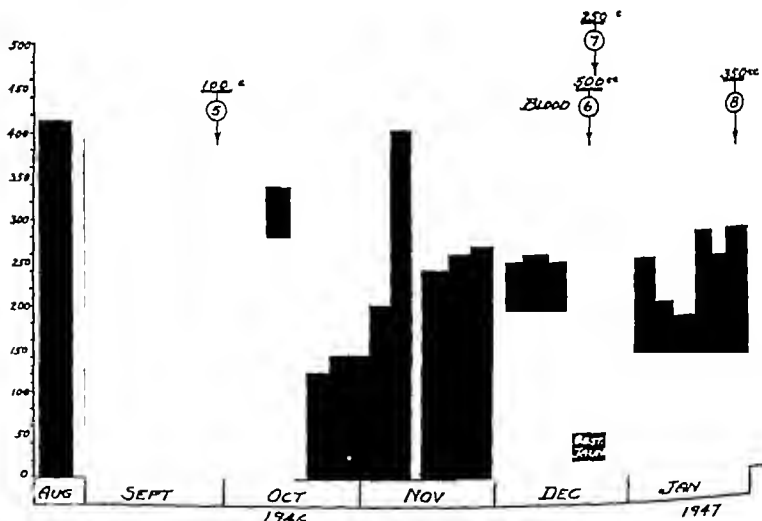


FIG 2.—UROBILINOGEN EXCRETION CASE I A.M. PERIOD II Vertical axis indicates urobilinogen excretion mg per day Note (1) variable effect following transfusions (2) spontaneous cyclic variations

It is thus clear that in each of these 4 cases we were able to confirm the observation of Josephs that the administration of plasma will induce a temporary reduction in fecal urobilinogen excretion in sickle cell anemia. This reduction was not, however, accompanied by any rise in the levels of hemoglobin or erythrocytes. Its possible significance will be discussed below.

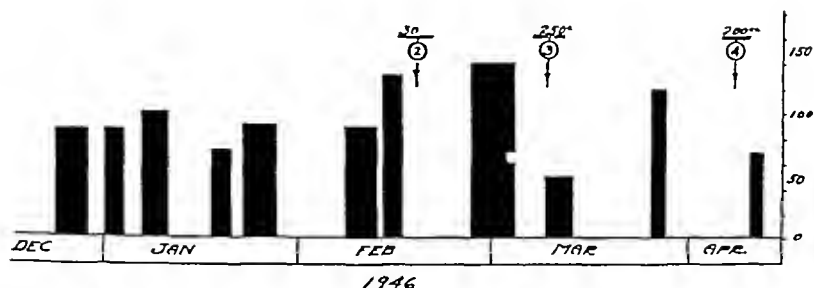


FIG. 1—(Continued)

## REVERSAL OF THE JOSEPHS PHENOMENON

Case I (A.M.) was followed closely for an additional period of twenty months (April 1946 to January 1948), during which time periodic observations were made on the relation of plasma transfusions to the urobilinogen output. A number of

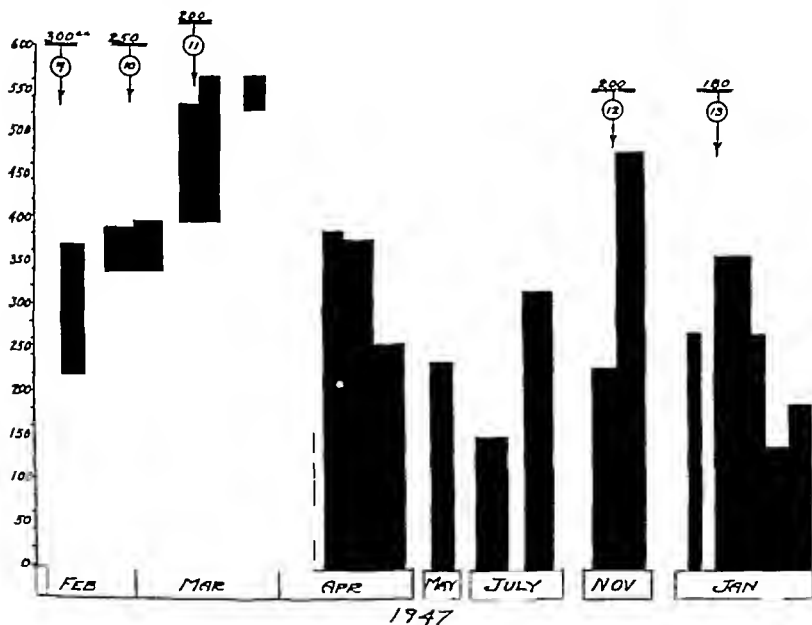


FIG. 3—UROBILINOGEN EXCRETION CASE I (A.M.) PERIOD III. Vertical axis indicates urobilinogen excretion mg per day. Note increased excretion following transfusions.

interesting features arose during this period. There were cyclic variations in urobilinogen output which could not be related to season or infection and were not accompanied by erythroblastosis. There were also mild intermittent attacks of abdominal pain which could not be attributed to increased hemolysis. In December 1946, there occurred a sudden attack of obstructive jaundice with intense icterus, a

direct van den Bergh reaction and clay colored stools, this was associated with fever, leukocytosis, extreme erythroblastosis, and severe abdominal pain. Exploratory laparotomy was considered but was not carried out because of amelioration of the symptoms.

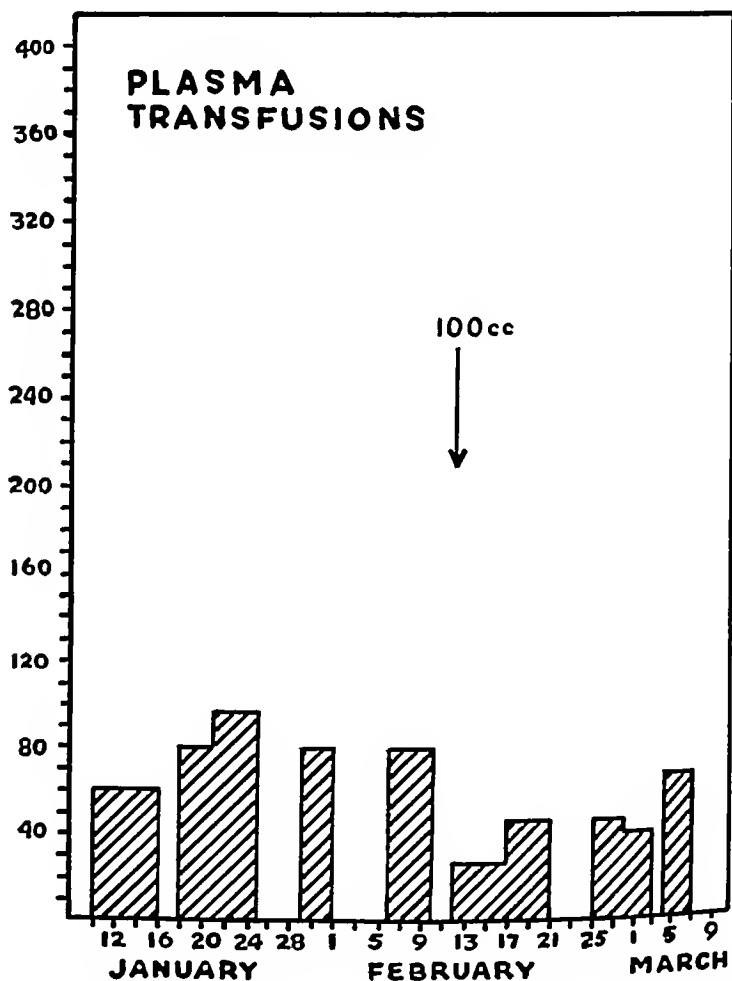


FIG. 4.—UROBILINOGEN EXCRETION CASE II W. M. Vertical axis indicates urobilinogen excretion mg per day. Horizontal axis indicates days. Note decreased excretion following transfusion.

tion of the symptoms. The obstruction was attributed to biliary sand, which has been known to produce occlusion of the bile passages in sickle cell<sup>6</sup> and other hemolytic anemias.

Of the greatest interest during this twenty month period of observation was the

change in the response to plasma transfusions. Nine such transfusions were given during this period. Between May 1946 and February 1947, four transfusions were given which produced virtually no change in urobilinogen output (fig 2). In

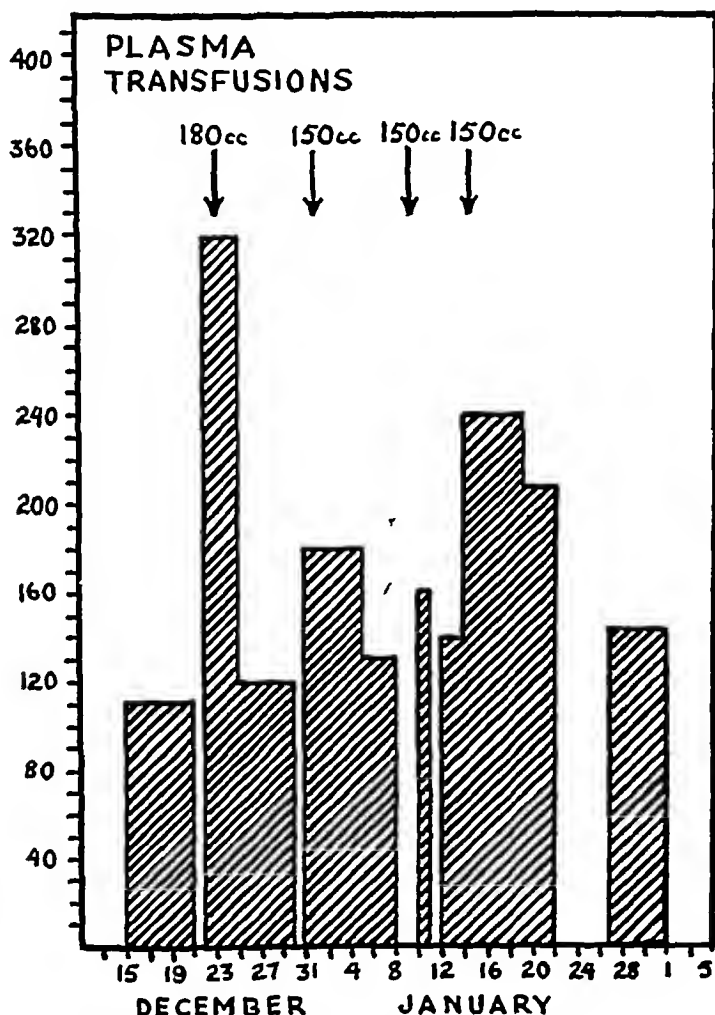


FIG 5—UROBILINOGEN EXCRETION CASE V R P Vertical axis indicates urobilinogen excretion mg per day Horizontal axis indicates days Note increased excretion following transfusion

the succeeding period of one year (February 1947 to February 1948), five more transfusions were given. Not only did these fail to decrease urobilinogen output but they actually produced the reverse effect—an increase in its excretion (fig 3). This, too, was not accompanied by any consistent change in the levels of



# THE EFFECT OF QUANTITATIVE AND QUALITATIVE PROTEIN DEFICIENCY ON BLOOD REGENERATION I WHITE BLOOD CELLS

By K. GUGGENHEIM, M D, AND EDITH BUECHLER, M Sc

THE LEVEL of dietary protein has been demonstrated by several investigators to be a factor in the regeneration of leukocytes and granulocytes. The studies of Kornberg et al.,<sup>1</sup> Wright and Skeggs<sup>2</sup> and of Daft<sup>3</sup> have shown, that diets of low protein content produce leukopenia and granulocytopenia, and that this abnormality can be effectively corrected by the administration of proteins or of the ten essential amino acids. Wissler<sup>7</sup> has noted, that protein depleted rabbits and rats exhibit a lowered granulocytic response following infection.

The studies outlined in this paper were carried out for the purpose of obtaining additional information concerning the effect of the level of dietary protein on the regeneration of leukocytes and granulocytes in protein depleted rats. Furthermore, the specific effects of various food proteins on the production of leukocytes and granulocytes were studied.

## METHODS

For the production of leukopenia, male albino rats within one week after weaning were fed a protein-free basal diet which consisted of 91 Gm starch, 5 Gm olive oil, and 4 Gm salt mixture, 0.1 mg thiamine hydrochloride, 0.2 mg riboflavin, 0.1 mg pyridoxin, 1.6 mg calcium pantothenate, 0.25 mg folic acid, and 100 mg choline chloride per 100 Gm ration were incorporated into the diet. Each rat received 100 I U vitamin A and 4 I U vitamin D twice weekly. After being fed on this diet for two weeks, leukopenia and granulopenia were noted in about 75 per cent of the animals. Leukopenia was considered to be present when the white blood cells numbered 4000 or less cells per cu mm, granulocytopenia when the number of granulocytes amounted to 1200 or less per cu mm. The granulocytopenia observed was not caused by secondary folic acid deficiency as in the experiments described by Wright and Skeggs<sup>2</sup> and by Daft<sup>3</sup> since additional supplementation of the diet with this vitamin did not delay the development of the blood dyscrasia. The hematologic data obtained from 55 normal and 100 protein depleted rats selected at random are listed in table 1.

The leuko- and granulocytopenic rats were used for the determination of the effectiveness of different levels of casein and of various food proteins on the production of white blood cells.

In order to test the effect of different levels of dietary protein the diets listed below were used (grams per 100 grams ration).

	C <sub>1</sub>	C <sub>2</sub>	C <sub>3</sub>	C <sub>18</sub>	C <sub>20</sub>
Casein	3	6	9	18	30
Rice starch	88	85	82	73	61
Olive oil	5	5	5	5	5
Salt mixture	4	4	4	4	4

These diets were supplemented with the above mentioned quantities of vitamins.

In the experiments with qualitative protein deficiency the following protein sources were used: egg powder, dried meat, casein, processed soya bean flour, peanut meal, maize flour, wheat flour (white) and gelatin. Egg powder, dried meat, soya bean flour and peanut meal were fat-extracted. The diets were prepared in the following manner: the various protein sources were incorporated in the protein-free

From the Department of Hygiene and Bacteriology, The Hebrew University, Jerusalem

basal diet by replacing an appropriate amount of starch so as to make the protein level of each diet 9 Gm per 100 Gm ration

Total white blood cell and granulocyte counts were made in the usual manner

After the leuco- and granulocytopenic rats were placed on the experimental diets white blood cell and granulocyte counts were carried out on the fourth eighth and fifteenth days respectively. As the numbers of leukocytes and granulocytes after two weeks were almost the same as after one week, the figures of the latter count are omitted

TABLE 1—Number of Leukocytes and Granulocytes in Normal and Protein Depleted Rats

		Leukocytes	Granulocytes	
			Number	Per cent of leukocytes
Normal	Mean	7970	2510	32
	$\sigma$	2720	1150	10.6
	$\epsilon$	368	155	1.4
Protein depleted	Mean	2420	690	31
	$\sigma$	805	265	10.2
	$\epsilon$	80.5	26.5	1.0

TABLE 2—The Effects of Diets Containing Different Levels of Casein, Given to Protein Depleted Rats on Changes in Leukocyte and Granulocyte Counts Means and Standard Errors

Diet	Manner of feeding	No. of rats	Fourth day			Eighth day		
			Weight grams	Leukocytes	Granulocytes	Weight, grams	Leukocytes	Granulocytes
				Per cu mm			Per cu mm	
C <sub>3</sub>	ad libitum	20	+1±0.8	-310±179	-340±79	+1±0.8	-1125±150	-510±67
C <sub>6</sub>	ad libitum	25	+3±0.5	+450±191	+40±79	+5±1.1	+910±248	+250±98
C <sub>9</sub>	ad libitum	23	+7±0.9	+1140±263	+400±30	+13±1.4	+1820±240	+740±137
C <sub>18</sub>	ad libitum	20	+10±0.6	+2630±300	+1130±146	+19±1.0	+4370±500	+1660±220
C <sub>18</sub>	Controlled	16	+7±1.0	+1500±330	+510±168	+14±1.6	+2050±302	+1030±267
C <sub>20</sub>	Controlled	17	+10±0.9	+3180±473	+1280±193	+11±0.9	+4980±244	+1870±244

## RESULTS

The effect of quantitative protein deficiency on regeneration of white blood cells. In this series of experiments the effect of various levels of dietary protein on regeneration of leukocytes were studied. Four diets, C<sub>3</sub>, C<sub>6</sub>, C<sub>9</sub> and C<sub>18</sub> were offered *ad libitum* to protein depleted rats. The changes obtained in weight and in total white blood cell and granulocyte counts are shown in table 2.

As can be seen from table 2, low protein diets induced a slight increase only and occasionally a further decrease in leukocyte and granulocyte numbers. Diet C<sub>18</sub>

on the other hand, which contained sufficient quantities of protein, caused in a four day period when given *ad libitum* a considerable increase in white blood cells which reached the normal number after seven days feeding. In the observed decreases and increases of leukocytes, granulocytes participated to a greater degree than lymphocytes and monocytes, the average percentage of granulocytes in total white blood cells decreased with  $C_2$  from 28 to 15, it remained constant (30) with  $C_6$ , with  $C_9$  and  $C_{18}$  slight increases were observed (from 25 to 30 and from 30 to 34, respectively). It seems, therefore, that granulocytes exhibit a greater sensitivity to protein intake than lymphocytes and monocytes. Statistical analysis of the changes in total white blood cells and granulocytes, observed after one week, showed that the casein levels of the four diets employed differed one from another to a highly significant degree (table 3).

TABLE 3—Statistical Analysis of Changes in Leukocyte and Granulocyte Counts Observed after One Week on Specified Diets. Probability that the Differences are due to Chance

Diets compared	Leukocytes	Granulocytes
$C_2$ vs $C_6$	0.01	0.01
$C_3$ vs $C_9$	0.01	0.01
$C_2$ vs $C_{18}$ <i>ad libitum</i>	0.01	0.01
$C_6$ vs $C_9$	0.01	0.01
$C_6$ vs $C_{18}$ <i>ad libitum</i>	0.01	0.01
$C_9$ vs $C_{18}$ <i>ad libitum</i>	0.01	0.01
$C_{18}$ <i>ad libitum</i> vs $C_{18}$ controlled	0.01	0.05
$C_3$ vs $C_{18}$ controlled	0.01	0.01
$C_{18}$ controlled vs $C_{30}$ controlled	0.01	0.0

Since the four above mentioned diets were offered *ad libitum*, the rats receiving the protein low diets ate considerably less than those receiving  $C_{18}$ . The food consumption of the rats fed  $C_3$  amounted to 60 per cent only of that of the rats given  $C_{18}$ . The observed effect of the diets low in casein may, therefore, be due to protein deficiency or to caloric deficiency or to both protein and caloric deficiency. In order to investigate this question the following series of experiments was conducted. One group of rats received  $C_{18}$  with controlled intake, i.e., an amount of food as was consumed by  $C_3$ , a second group was fed a protein rich diet, containing 30 per cent casein ( $C_{30}$ ) also given with controlled intake. These rats received, therefore, the same amount of calories as  $C_3$  and the same quantity of protein as the rats which were allowed to eat  $C_{18}$  *ad libitum*. The results obtained and their statistical treatment are shown in tables 2 and 3.

These tables demonstrate that the increase in leukocyte and granulocyte counts obtained with  $C_{18}$ , given in restricted amounts is considerably lower than that observed with  $C_{18}$  offered *ad libitum*. On the other hand, this diet proved to be statistically significantly superior to  $C_3$ , although there was no difference in the caloric intake of these two groups. Furthermore, the rats receiving  $C_{30}$  in restricted amounts showed a similar response as those receiving the same quantity of protein

( $C_{18}$  *ad libitum*), and a statistically significantly superior response than those receiving  $C_{18}$  in restricted amounts

The latter observation suggests that the amount of protein eaten, and not its level in diet, is important for the regeneration of white blood cells. The highly significant difference in the response between  $C_3$  and  $C_{18}$ , given in restricted amounts, does not contradict this interpretation. In caloric deficiency the organism is forced to divert protein from cell-synthesis to energy production. When the caloric supply is insufficient, it is immaterial to the caloric economy of the animal whether the restricted diet is rich or poor in protein. The decisive role of the protein intake for white cell regeneration is clearly shown by a comparison of the increases reached by  $C_{18}$  and  $C_{20}$ , both given in restricted amounts. Rats receiving  $C_{20}$  exhibit a significantly larger increase of white cells than those fed on the same

TABLE 4.—*The Effect on Increase in Leukocyte and Granulocyte Counts of Diets Containing Various Proteins at 9 per cent Level Given ad libitum to Protein Depleted Rats Means and Standard Errors*

Source of protein	No of rats	Fourth day			Eighth day		
		Weight grams	Leukocytes	Granulo cytes	Weight, grams	Leukocytes	Granulo cytes
Egg	22	10±0 4	3320±151	1790±137	20±0 4	4300±188	1880±203
Meat	17	10±1 0	3260±150	1540±114	18±1 1	4120±290	1870±204
Peanut	19	1±0 8	1350±207	750±140	4±1 1	1980±194	890±124
Soya	23	7±0 6	1800±156	900±110	14±0 8	1870±163	850±126
Casein	23	7±0 9	1140±263	400±30	13±1 4	1820±240	740±737
Wheat	19	3±0 7	690±162	580±124	3±0 8	790±182	500±156
Gelatin	19	-1±0 6	620±239	640±120	-2±0 9	610±182	480±105
Maize	19	1±0 7	340±190	230±108	1±1 1	500±207	340±105

amounts of  $C_{18}$ . It may, therefore, be concluded, that protein intake plays a decisive role in white blood cell regeneration, and that this effect is due to be masked in a protein-high diet ( $C_{18}$ ), when given in insufficient amounts.

*The effect of qualitative protein deficiency on regeneration of white blood cells.* In our second series of experiments the effects of various proteins (egg, meat, peanut, soya, casein, wheat, gelatin, maize—fed *ad libitum* at 9 per cent level) on production of white blood cells in protein depleted rats were compared. The results are shown in table 4.

It follows from table 4, that protein quality as well as quantity determines white blood cell regeneration. Among the food proteins investigated the proteins of egg and meat rank first. Both are similar in this respect, and, given at 9 per cent level, they exhibited an effect similar to that of casein fed at 18 per cent level. Casein, peanut and soy bean protein rank next. It is interesting to note, that peanut protein effects a similar degree of white blood cell regeneration as does soy bean and casein, despite its decidedly inferior effect on growth. Gelatin, wheat and maize proteins are the least effective for white blood cell regeneration as well as in their growth promoting efficiency. The reactions elicited by diets containing

9 per cent of these proteins are similar to those produced by casein at 6 per cent level

Statistical treatment of the differences in mean increases of total white blood cells and of granulocytes, obtained after one week, revealed the following facts

*Total white blood cells* Egg or meat vs each of the other proteins tested differences highly significant (probability of the occurrence of the observed mean difference in a random sample 1 100 or less) Peanut or casein or soya vs wheat or gelatine or maize differences highly significant (probability 1 100 or less) The differences within each of the three groups were not found to be significant

*Granulocytes* Egg or meat vs each of the other proteins difference highly significant (probability 1 100 or less) Peanut vs wheat difference significant (probability 1 20) Peanut vs gelatin or maize difference highly significant (probability 1 100 or less) Soya vs wheat difference not significant Soya vs gelatin difference significant (probability 1 20) Soya vs maize difference significant

TABLE 5—Per cent of Granulocytes and of Lymphocytes and Monocytes in Protein Depleted Rats before and on the Fourth and Eighth Days after Feeding Various Proteins at 9 per cent Level

Source of protein	Protein depleted		Fourth day		Eighth day	
	Granulocytes	Lymphocytes	Granulocytes	Lymphocytes	Granulocytes	Lymphocytes
Egg	32	68	44	56	40	60
Meat	29	71	38	62	38	62
Peanut	28	72	36	64	35	65
Soya	33	67	38	62	37	63
Casein	25	75	28	72	30	70
Wheat	30	70	43	57	40	60
Gelatin	25	75	40	60	36	64
Maize	29	71	32	68	35	65

(probability 1 100) Casein vs wheat or gelatin difference not significant Casein vs maize difference significant (probability 1 50) The difference within each of the three groups were not found to be significant

It is noteworthy, that all proteins tested produced an increase in granulocytes which was accompanied by a relative decrease in lymphocytes and monocytes Details are given in table 5 The data shown in table 5 suggest that granulocytes regenerate more quickly after protein feeding than lymphocytes and monocytes These observations confirm the above mentioned fact that the percentage of granulocytes decreased with  $C_3$ , remained constant with  $C_4$  and increased with  $C_{11}$

#### DISCUSSION

Our results demonstrate that protein deficient diets invariably impair the regeneration of white blood cells in protein-depleted rats Normal regeneration will occur only when diets containing quantitatively and qualitatively optimal proteins are administered A diet, however, containing an optimal level of protein ( $C_{11}$ ), but given in restricted amounts, will not promote optimal regeneration of

leukocytes. In this masked form of protein deficiency food protein is utilized for energy, and is insufficient therefore for purposes of cell synthesis. A similar phenomenon has already been described by Kosterlitz and Campbell<sup>6</sup> studying the effect of protein deficiency on liver cytoplasm as well as in our studies<sup>3</sup> on the effect of protein deficiency on the bacteriocidal properties and phagocytic activity of peritoneal fluid.

The effect of various food proteins on regeneration of white blood cells corresponds, more or less, to their growth promoting efficiency. Only peanut protein seems to be an exception to this rule. Its relative efficiency on white blood cell production was found to be greater than its growth promoting quality. Since the amino acid composition of each protein determines its nutritive value and growth promoting efficiency (Block and Mitchell<sup>1</sup>), it may be concluded, that, generally speaking, the same amino acid makeup is necessary for both white blood cell production and for growth. Experiments designed to study this question further are in progress.

#### SUMMARY

1. The effect of diets, varying in quantity or quality of protein, on white blood cell regeneration was studied in leukopenic rats, the leukopenia having been induced by a protein-free diet.

2. Diets containing different amounts of casein (3, 6, 9 and 18 per cent, respectively), were fed *ad libitum*. At the 3 per cent level, a further decrease occurred of white blood cells, whereas the other three diets initiated a regeneration of leukocytes, its degree being more or less in proportion to the casein content.

3. In experiments with diets containing 18 and 30 per cent of casein, the amount of protein eaten and not its level in diet was the decisive factor in the regeneration of leukocytes. The white blood cell regenerating effect of a diet containing an optimal level of protein, may be neutralized when given in restricted amounts.

4. Diets containing nutritionally inferior proteins, fed at 9 per cent level, also impaired normal regeneration of leukocytes. The white blood cell regeneration afforded by the proteins investigated was found to increase in the following order: maize, gelatin, wheat, casein, processed soya, peanut, meat, egg.

5. In white blood cell regeneration promoted by dietary protein, granulocytes were found to react to a greater degree than lymphocytes and monocytes.

#### REFERENCES

- <sup>1</sup> BLOCK R. J. AND MITCHELL, H. H. *Nutr. Abstr. Rev.* 16: 249, 1946-1947.
- <sup>2</sup> DAFT F. S. *Fed. Proc.* 6: 405, 1947. *Pub. Health Rep.* 62: 1785, 1947.
- <sup>3</sup> GUGGENHEIM K. AND BUECHLER E. *J. Immunol.* 54: 349, 1946.
- <sup>4</sup> KORNBERG A. *J. Biol. Chem.* 164: 203, 1946.
- <sup>5</sup> — DAFT F. S. AND SEBRELL, W. H. *Science* 103: 646, 1946.
- <sup>6</sup> KOSTERLITZ H. W. AND CAMPBELL R. M. *J. Physiol.* 104: 16P, 1945.
- <sup>7</sup> WISLER R. W. *J. Inf. Dis.* 80: 250, 164, 1947.
- <sup>8</sup> WRIGHT L. D. AND SKEGGS H. R. *Proc. Soc. Exp. Biol. & Med.* 63: 327, 1946.

# THE EFFECT OF QUANTITATIVE AND QUALITATIVE PROTEIN DEFICIENCY ON BLOOD REGENERATION II HEMOGLOBIN

By EDITH BUECHLER, M Sc , AND K GUGGENHEIM, M D

SUFFICIENT evidence is present to indicate that the feeding of rats on a diet containing an inadequate amount of protein<sup>9, 11</sup> or lacking one or more of the ten essential amino acids<sup>12</sup> (especially tryptophane,<sup>1, 2, 5, 17</sup> lysine<sup>4, 6</sup> isoleucine,<sup>10</sup> histidine<sup>3, 16</sup> and phenylalanine<sup>8</sup>) will result in the development of a mild to moderate chronic anemia. The problem of the comparative value of diets containing different levels of protein or various commonly consumed dietary proteins for hematopoiesis is raised by the foregoing studies. The present paper reports experiments with rats, designed to determine (1) the effect of various levels of dietary protein on hemoglobin regeneration in protein depleted rats, (2) the role played by various animal and vegetable proteins in enhancing the regeneration of hemoglobin.

## METHODS

For the production of anemia male albino rats weighing 150-250 Gm were fed the protein-free basal diet described in the preceding paper<sup>14</sup> over a period of eight to ten weeks. During this time the weight of the rats decreased by 28 to 35 per cent and a moderate anemia developed. The results of hemoglobin determinations of 50 normal and 100 protein depleted rats selected at random are shown in table 1.

The anemic rats were used for the determination of the effectiveness of different levels of casein and of various food proteins on the formation of hemoglobin.

The diets employed were the same as those described in our preceding paper.

Hemoglobin was determined in the tail blood by using the acid hematin method.

After placing the anemic rats on the experimental diets hemoglobin determinations were carried out on the 11th and 21st days.

## RESULTS

*The effect of quantitative protein deficiency on hemoglobin regeneration.* In the first part of our investigation, diets containing 0, 3, 9, and 18 per cent casein, respectively, were given *ad libitum* to protein-depleted rats. The results obtained after ten and twenty days are shown in table 2.

The table demonstrates that weight recovery as well as hemoglobin regeneration is dependent on the protein content of the diet. The protein-free diet causes a further decrease in weight and in hemoglobin concentration, protein low diets promote slight increases, whereas a diet with a sufficient level of casein causes a quick recovery of weight and a considerable regeneration of hemoglobin. Statistical analysis of the changes in hemoglobin concentration observed after three weeks showed that the differences between the four diets employed are highly significant (table 3).

The differences obtained with the three protein diets are due to the varying amounts of protein eaten and not to a diminished food intake with the low protein diets. Contrary to the findings with young rats, described in our preceding paper,<sup>14</sup>

From the Department of Hygiene and Bacteriology The Hebrew University Jerusalem

adult rats receiving the C<sub>2</sub> diet consumed as much food (about 15 grams per day) as those offered the C<sub>18</sub> diet. In order to test a possible effect that caloric restriction

TABLE 1—Hemoglobin Content of the Blood of Normal and of Protein Depleted Rats

		Hemoglobin Gm per 100 Gm
Normal rats	Mean	14.4
	$\sigma$	2.20
	$\epsilon$	0.31
Protein depleted rats	Mean	8.5
	$\sigma$	1.37
	$\epsilon$	0.14

TABLE 2.—The Effect of Changes in Weight and in Concentration of Hemoglobin of Diets Containing Various Levels of Casein Given to Protein Depleted Rats Means and Standard Errors

Level of casein %	Intake	No of rats	11th day		21st day	
			Weight grams	Hemoglobin %	Weight, grams	Hemoglobin %
0	ad libitum	12	-11±1.3	-0.5±0.30	-22±1.5	-1.5±0.50
3	ad libitum	12	+1±1.5	+0.3±0.30	+6±2.1	+0.3±0.27
9	ad libitum	15	+21±2.2	+0.6±0.20	+43±4.7	+1.4±0.18
18	ad libitum	14	+26±2.8	+1.7±0.18	+51±6.8	+2.2±0.11
9	Controlled	12	+11±2.1	+1.0±0.23	+22±3.6	+1.5±0.21
18	Controlled	14	+12±3.2	+1.4±0.35	+26±2.9	+2.0±0.43
30	Controlled	11	-1±2.2	+1.1±0.46	-4±4.6	+1.4±0.30

TABLE 3—Statistical Analysis of Changes in Weight and in Concentration of Hemoglobin Observed after Three Weeks on Specified Diets Probability that the Differences Are due to Chance

Diets compared	Weight	Hemoglobin
Protein free vs C <sub>2</sub> ad libitum	0.01	0.01
Protein free vs C <sub>8</sub> ad libitum	0.01	0.01
Protein free vs C <sub>18</sub> ad libitum	0.01	0.01
C <sub>2</sub> ad libitum vs C <sub>8</sub> ad libitum	0.01	0.01
C <sub>2</sub> ad libitum vs C <sub>18</sub> ad libitum	0.01	0.01
C <sub>8</sub> ad libitum vs C <sub>18</sub> ad libitum	0.3	0.01
C <sub>2</sub> ad libitum vs C <sub>8</sub> controlled	0.01	0.7
C <sub>18</sub> ad libitum vs C <sub>18</sub> controlled	0.01	0.7
C <sub>18</sub> controlled vs C <sub>20</sub> controlled	0.01	0.3
C <sub>18</sub> ad libitum vs C <sub>20</sub> controlled	0.01	0.02

may exert, we fed to two series of rats our diets C<sub>2</sub> and C<sub>18</sub>, which were given in quantities amounting to 60 per cent only, i.e., 9 grams per day, of the amount of food eaten when offered *ad libitum*. As may be seen from tables 2 and 3 this dietary



regimen, although retarding weight recovery, elicited a similar response in hemoglobin regeneration as the same diets when given *ad libitum*. It seems, therefore, that in caloric deficiency the body utilizes dietary protein first for hemoglobin formation, which evidently has a priority over weight recovery.

In a third experiment, protein-depleted rats were given a 30 per cent casein diet, also in controlled amounts. These animals received, therefore, the same quantity of protein as those given  $C_{18}$  *ad libitum* and the same amount of calories as the rats fed on  $C_{18}$  in restricted amounts. Table 2 shows a marked increase in hemoglobin concentration, whereas the effect on weight recovery proved to be deleterious. It may be that such an ill-balanced diet causes serious disturbances in the protein economy of the body, leading to the observed phenomena. Further studies in this

TABLE 4.—*The Effect of Change in Weight and in Concentration of Hemoglobin of Diets Containing Various Proteins at 9 per cent Level, Given in Controlled Amounts to Protein Depleted Rats*  
Means and Standard Errors

Source of protein	No of rats	11th day		21st day	
		Weight grams	Hemoglobin %	Weight, grams	Hemoglobin, %
Egg	11	+21±1.7	+1.1±0.27	+29±2.3	+2.4±0.42
Meat	12	+16±1.9	+1.1±0.31	+27±2.3	+2.2±0.30
Soya	12	+9±1.7	+0.9±0.28	+15±2.2	+2.0±0.35
Casein	12	+11±2.1	+1.0±0.23	+22±3.6	+1.5±0.21
Peanut	11	+2±1.2	+0.3±0.17	+3±2.4	+1.3±0.39
Maize	12	-1±0.8	+0.7±0.19	+1±2.1	+1.3±0.31
Wheat	10	+4±1.1	+0.1±0.37	-1±0.9	+0.4±0.37
Gelatin	14	-16±2.7	-0.2±0.21	-29±3.6	-0.5±0.35

direction are in progress. Comparing the effect of this diet to those of  $C_{18}$ , given either *ad libitum* or in restricted amounts, it will be seen from table 3, that  $C_{30}$  given in restricted amounts causes a smaller increase of hemoglobin concentration than  $C_{18}$ , the difference between  $C_{30}$  and  $C_{18}$  given *ad libitum*, however, was found to be statistically significant, thus again showing that restriction of the amount of food protein interferes more seriously with the regeneration of hemoglobin than restriction of calories.

*The effect of qualitative protein deficiency on hemoglobin regeneration.* In the second part of our study the effects of various food proteins (egg, meat, processed soya, casein, peanut, maize, wheat, gelatin) on hemoglobin regeneration were compared. Because the rats receiving gelatin, peanut, wheat and maize proteins are considerably less than those receiving the other proteins, the food intake of all rats was equalized and restricted to 9 grams per day. The results obtained after ten and twenty days are tabulated in table 4.

As can be seen from table 4, the different food proteins tested exert different effects on hemoglobin formation. The most effective proteins are those of eggs, meat and soya, in three weeks they elicit an increase of 2 per cent or more in hemoglobin concentration and at 9 per cent level they are as effective as casein at 18

per cent level Casein, peanut and maize proteins rank next. With these proteins increases of 1.3 to 1.5 per cent were observed. The least efficacious proteins were found to be those of wheat and gelatin. They cause a negligible increase only and sometimes a decrease in hemoglobin concentration. The effects of the various food proteins on hemoglobin regeneration correspond, more or less, to their effects on weight recovery. Only casein and soya represent exceptions, the former was found to be more potent in promoting weight recovery than hemopoiesis whereas soya protein was found to be more efficient with respect to hemoglobin formation than in its weight regaining capacity.

### DISCUSSION

The foregoing results indicate that diets containing insufficient amounts of protein will not support normal weight recovery and hemoglobin regeneration in

TABLE 5—*The Relative Values of Various Food Proteins for Regeneration of Hemoglobin and of Granulocytes, Egg Proteins Assumed to be 100*

Source of protein	Regeneration of	
	Hemoglobin	Granulocytes
Egg	100	100
Meat	92	99
Soya	83	45
Casein	62	39
Peanut	54	47
Maize	54	18
Wheat	17	27
Gelatin	—2.1	26

protein-depleted anemic rats. Furthermore, low caloric intake, sufficient to suppress normal weight recovery, causes no reduction in the formation of hemoglobin. These findings confirm those of other authors made with different experimental techniques. Albanese et al.,<sup>1</sup> who fed normal rats on low caloric diet sufficient to inhibit growth, did not observe a reduction of hemoglobin concentration. Whipple<sup>14</sup> and Whipple, Miller and Robschert-Robbins<sup>15</sup> studying hemoglobin regeneration in dogs made anemic by the withdrawal of blood, concluded that hemoglobin stands apart in the protein economy of the body in that it does not contribute freely to the protein pool, as do other tissue proteins. Under conditions of protein fasting the body will give up large amounts of protein from its organs to produce hemoglobin, which has a priority over the tissue and organ proteins.

The comparative value of dietary proteins for hemopoiesis in the rat has already been studied with some proteins by Orten and Orten.<sup>12</sup> At an 18 per cent protein level, casein, lactalbumin, dried skim milk, and a mixture of dried skim milk and dried beef blood proved to be of about the same value, both for hemoglobin maintenance in the growing rat and for hemoglobin regeneration in the adult animal. On the other hand, dried beef blood proteins were found to be inferior. In our experiments in which the different proteins were fed at 9 per cent level we found

considerable differences in the hematopoietic values of the eight proteins investigated

The nutritive value of each protein tested is not the same for production of hemoglobin and of granulocytes, as can be seen by comparing the results of the present two studies. Assuming the relative value of egg proteins, as obtained after three weeks for hemoglobin and after one week for production of granulocytes, to be 100, the relative values of the proteins tested can be seen in table 5.

It follows from table 5, that the nutritional values of casein and soya and maize proteins are considerably higher for hemoglobin formation than for production of granulocytes, whereas those of wheat protein and gelatin are much lower.

### SUMMARY

1 The effect of diets, varying in quantity or quality of protein, on hemopoiesis was studied in protein depleted and anemic adult rats.

2 In experiments with diets containing different amounts of casein (0, 3, 9 and 18 per cent, respectively), and fed *ad libitum*, it was found that with a protein free diet a further decrease of hemoglobin occurred, whereas the other three diets initiated a regeneration of hemoglobin, its degree being more or less proportional to the casein content.

3 In experiments, in which diets with 9 and 18 per cent of casein, respectively, were given in restricted amounts, it was found that the degree of hemoglobin formation was similar to that with the same diets when given *ad libitum*, whereas the weight gain was considerably less. It is concluded, therefore, that in caloric deficiency hemoglobin formation has a priority over weight recovery.

4 A diet containing 30 per cent casein and given in restricted amounts induced a further weight loss, whereas the concentration of hemoglobin showed a marked increase. Comparing the results obtained by this diet with those observed with 18 per cent casein diets, given either *ad libitum* or in controlled amounts, it was evident that restriction of the quantity of food protein interferes more seriously with hemopoiesis than restriction of calories.

5 Diets containing nutritionally inferior proteins fed at 9 per cent level, also impaired normal hemopoiesis. Hemoglobin regeneration induced by the proteins investigated was found to decrease in the following order: eggs, meat, processed soya, casein, peanut, maize, wheat, gelatin.

6 Comparing the nutritive value of various proteins for regeneration of hemoglobin and of granulocytes it was found, that casein and soya and maize proteins are considerably more efficient for hemoglobin formation than for production of granulocytes, whereas wheat protein and gelatin have a higher granulocytopenic capacity.

### REFERENCES

- <sup>1</sup> ALBANESE A. A., HOLT L. E., KAJDI C. AND FRANKSTON J. E. J. Biol. Chem. 148: 299, 1943.
- CHIN K. S. Zeitschr. physiol. Chem. 257: 18, 1938.
- <sup>2</sup> FONTES G. AND TRIVOLLE L. Sang. 4: 658, 1930.
- <sup>3</sup> GILLISPIE M., NEUBERGER A. AND WEBSTER T. A. Biochem. J. 39: 203, 1945.

- <sup>13</sup> GUGGENHEIM K., AND BUECHLER EDITH The effect of quantitative and qualitative protein deficiency on blood regeneration I White blood cells Blood this issue pag- 958
- <sup>14</sup> HAMADA T Ztschr physiol Chem 243 258 1936
- <sup>15</sup> HARRIS H A., NEUBERGER A., AND SANDER F Biochem J 37 508, 1943
- <sup>16</sup> HOGAN, A G POWELL, E L. AND GUERRANT R E J Biol Chem 137 41, 1941
- <sup>17</sup> MAUN M E CAHILL W M., AND DAVIS R M Arch Path 39 294 1945
- <sup>18</sup> METCOFF J FAVOUR C B AND STARE F J J Clin Investigation 24 82, 1945
- <sup>19</sup> ORTEN J M. BOURQUE J E AND ORTEN A U J Biol Chem 160 435 1945
- <sup>20</sup> ORTEN A U AND ORTEN J M. J Nutr 26 21 1943
- <sup>21</sup> — AND — J Nutr 30 137 1945
- <sup>22</sup> —, AND — J Nutr 31 765 1946
- <sup>23</sup> WHIPPLE G H Am J M Sc 203 477, 1942.
- <sup>24</sup> — MILLER L. L AND ROBSCHT ROBBINS F S J Exp Med 85 277 1947
- <sup>25</sup> YESHODA K. M Curt Sci 14 77 1945
- <sup>26</sup> — AND DAMODARAN M Biochem J 41 382 1947

## HYPOPLASTIC ANEMIA DUE TO ATABRINE

B<sub>3</sub> ALFRED P. FISHMAN, M D , AND J. MURRAY KINSMAN, M D

IT IS the purpose of this paper to present the clinical picture which may follow the prolonged ingestion of atabrine. This picture is characterized by a severe anemia which may or may not be associated with a characteristic dermatitis. The dermatitis has been previously described<sup>1-6</sup>, the anemia warrants further consideration. Custer,<sup>7</sup> basing his conclusions on an analysis of biopsy and autopsy material forwarded from the southwest Pacific to the Army Institute of Pathology, indicated atabrine as the agent responsible for the production of the hypoplastic anemia in the cases he reviewed. His report, based on pathologic data, by its very nature stresses the gravity and the poor prognosis of the illness. However, our experience indicates that a more optimistic approach is warranted. We are reporting the pertinent data regarding 7 patients who developed anemia following the prolonged ingestion of atabrine for malarial suppression while serving in the southwest Pacific area. Four suffered a concomitant dermatitis (fig. 1). The majority recovered. This group of patients illustrates the course and prognosis of hypoplastic anemia due to atabrine.

Hypoplastic anemia indicates a disorder of the bone marrow characterized by diminished hematopoiesis. The anemia fails to respond to the usual methods of therapy other than whole blood transfusion. The degree of anemia is variable; leukopenia and granulocytopenia are invariably present. Thrombocytopenia is usually marked and is responsible for hemorrhagic phenomena. The bone marrow varies histologically in architecture, degree of cellularity and maturity.

### CLINICAL MATERIAL

Seven patients with hypoplastic (refractory) anemia were admitted to Moore General Hospital, a tropical disease center. In each instance the anemia was so severe as to require frequent transfusions of whole blood. Each patient exhibited hemorrhagic phenomena. All had spent several months in the southwest Pacific area, but their military itineraries within the area showed little duplication. Only 2 had served in the jungles. One was a nurse, one a Medical Officer, and the others were enlisted men in combat units. Six were white and one was a Negro. All had taken atabrine for many months. Final hospitalization was occasioned by the development of symptoms of anemia in two instances, the appearance of a skin eruption in four, and diarrhea and acute otitis media in one. Details are presented in table 1.

### ETIOLOGY

It is indicated in table 1, that 4 of the 7 patients were hospitalized primarily for dermatitis. This was either of a lichenoid (fig. 1) or an eczematoid type. It has been established that these lesions, as seen in patients from the Pacific area, are caused

From the Medical Service, Moore General Hospital, Swannanoa, N. C.

by prolonged atabrine therapy.<sup>16</sup> This form of dermatitis was not observed in natives or white residents who had never taken atabrine. Moreover, the dermatitis ceased to progress or disappeared entirely after atabrine was discontinued. In case 1 of this series (fig. 2), both dermatitis and a mild anemia appeared while the patient was in the southwest Pacific area. Upon his return to the United States he

TABLE 1—*History of Antecedent Drug Ingestion and Primary Reason for Hospitalization in 7 Cases of Refractory Anemia*

Case	Atabrine history	Reason for final hospitalization	Other drugs prior to onset of anemia
1—M. W. 33 infantryman	0.1 Gm daily for 7 mos none for 11½ mos except for 2 courses of 5 and 7 days each then 0.1 Gm daily for 3 mos when anemia appeared	Anemia	Sulfadiazine 18 mms and penicillin 12 mms previously
2—M. W. 26 infantryman	0.1 Gm daily for 14 mos when dermatitis appeared 6 weeks later anemia first discovered	Dermatitis (lichen planus)	None
3—M. W. 26 infantryman	0.1 Gm daily for 4 mos none for 2 mos 0.1 Gm daily for 6 mos none for 8 mos 0.1 Gm daily for 2½ mos 6 weeks later anemia first discovered	Anemia	None
4—M. W. 37 mechanic	0.1 Gm daily for 11 mos Discontinued because of dermatitis 3 mos before detection of anemia	Dermatitis (lichen planus)	None
5—F. W. 28 nurse	0.1 Gm daily for 18 mos Discontinued because of dermatitis 1 mo before detection of anemia	Dermatitis	Penicillin
6—M. W. 47 medical officer	0.1 Gm daily for 11 mos Discontinued upon evacuation to U S 7 days before detection of anemia	Recurrent diarrhea cause undetermined and acute Otitis Media	Sulfadiazine 6 Gm. daily for 9 days (4 mos before admission). Carbarsone 1.5 Gm for 1 day (4 mos before admission)
7—M. C. 32 infantryman	0.1 Gm daily for 8 mos Discontinued because of dermatitis 1 mo before detection of anemia	Dermatitis	None

discontinued taking atabrine and both the anemia and the dermatitis disappeared. Later, while still in the United States, he resumed atabrine medication for the suppression and treatment of recurrent malarial attacks; a severe anemia and dermatitis resulted. The anemia was ameliorated and the dermatitis improved by discontinuing atabrine.

It should be emphasized that the duration of atabrine therapy appeared to be the determining factor. Experience with the atabrine dermatides demonstrates that the drug must be ingested for comparatively long periods of time to produce

the eruption. In experiments which were done to reproduce the skin lesions it was found that they recurred only after the drug had been taken for several weeks.<sup>4</sup> In all 7 cases reported here, the patients had taken the drug for many months. The effect of the drug has been ascribed to idiosyncrasy, in an experimental study



FIG. 1.—CONCOMITANT (LICHENOID) DERMATITIS

by Parmer<sup>9</sup> no correlation could be established between the concentration of atabrine in the various blood cells and the cells predominantly affected by the hypoplastic anemia.

#### CLINICAL AND HEMATOLOGIC DATA

In table 2, the clinical and hematologic data are tabulated. It will be noted that in each instance anemia was present at the time of original examination, it was usually severe, although in one case (case 5) it was mild. The color index was

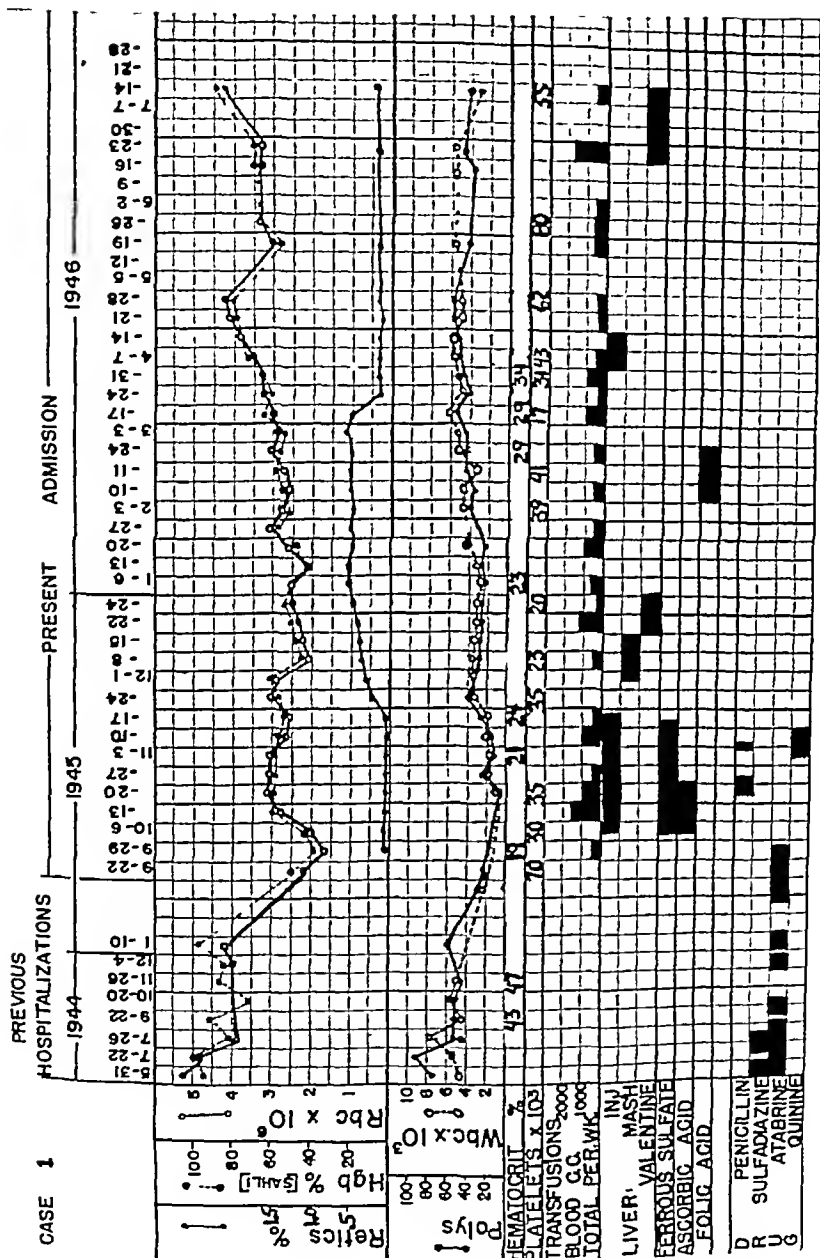


FIG. 2.—THE COURSE IN CASE 1.



usually above one. Macrocytosis was the rule. The volume of packed cells (hematocrit) was uniformly low. Leukopenia was invariably present with the polymorphonuclear percentage usually low. The thrombocytes were always below

TABLE 2.—*Clinical and Hematologic Status*

Case	Symptoms of bone marrow alteration	[Initial Hemogram]							Bone marrow early in disease
		RBC	Hgb (Sahli)	Cell vol	WBC	P	Platelets	Retic	
		mill	%			%		%	
1	Weakness dizziness headaches. Hemorrhages from gums throat nose	2.94	56	24	3,500	58	35,000	0.2	Absence of megakaryocytes
2	Dyspnea on exertion tachycardia weakness Hemorrhages in retina	1.15	25	13	3,450	32	25,900	1.3	Normal
3	Dizziness weakness blurring of vision Hemorrhages from gums and throat and into skin and retina hematuria	2.25	60	26	3,500	45	18,500	0.2	Hypocellular, occasional megakaryocyte
4	Fatigability, paresthesias of hands and feet tachycardia palpitation Hemorrhages from nose and into retina	1.83	42	17	1,750	38	17,000	0.9	Absence of megakaryocytes
5	Weakness dyspnea on exertion tachycardia Hemorrhages into skin and retina	4.1	80	—	4,750	22	29,000	0.7	Marked depression of all elements
6	Hemorrhages into skin and mucous membranes	2.47	46	21	3,200	54	20,000	0.2	Normal
7	Syncope stomatitis Hemorrhages from gums	1.91	42	19	2,050	12	13,000	—	Not done

40,000 per cu mm. With one exception, the reticulocyte count was less than 1 per cent, this patient recovered rapidly. Bone marrow studies were done on 6 patients: in 2 the examination was found to be normal, in 2 normal except for the absence of megakaryocytes, and in 2 there was marked hypoplasia of all elements.

In addition to the data incorporated in table 2, certain additional information is available. Fever was invariably present but was usually not marked. The spleen was barely palpable in only one instance, the lymph nodes were enlarged in two patients and at autopsy were described as hemolymph nodes. In only one patient was the coagulation time prolonged but the bleeding time was always prolonged. The tourniquet test was positive in all cases at the height of the illness. In every instance there was defective clot retraction. There was no evidence of increased

TABLE 3—*Therapy and Clinical Course*

Case	Therapy	Clinical Course	Outcome
1	Transfusions diet liver mash liver extract orally and hypodermically folic acid iron	Frequent hemorrhages from gums throat and nose gradually decreasing. Slow but steady improvement.	Recovered
2	Transfusions diet liver extract orally and hypodermically iron	Moderate steady reticulocytosis persisting when medication was discontinued.	Recovered
3	Transfusions diet liver extract orally and hypodermically iron sternal marrow transplant	Frequent hemorrhages from gums and throat which became uncontrollable hematuria purpura.	Died
4	Transfusions diet liver extract orally and hypodermically iron ascorbic acid hypodermically	No response for a long time. Later reticulocytosis to 10% following liver intramuscularly and maintained by liver mash by mouth. Then gradual improvement temporarily interrupted by homologous serum jaundice.	Recovered
5	Transfusions diet liver extract orally and hypodermically iron penicillin	Gradual improvement. Slow reticulocyte response not related to therapy.	Recovered
6	Transfusions diet liver extract hypodermically	Persistently downhill in spite of transfusions. Death following mastoiditis and acute endocarditis ( <i>Paracolon bacillus</i> ).	Died
7	Transfusions diet liver extract hypodermically penicillin pyridoxine	Hemorrhages stopped after first transfusion but no change in leukocyte or platelet deficiency. Death from staphylococcus (hemolytic) septicemia.	Died

\* Diet: High liver, high carbohydrate, high protein, high vitamin. Iron: Ferrous sulfate orally.

hemolysis. In 6 patients, gastric analysis was done and in no case was achlorhydria demonstrated.

In 4 patients, a skin eruption was present when the anemia was first detected. One patient was originally hospitalized because of severe diarrhea and acute otitis media but hemorrhagic phenomena quickly supervened and became the predominant symptom.

#### THE THERAPY AND CLINICAL COURSE

The broad outlines of the therapeutic program and the clinical course are summarized in table 3. Four of the 7 cases eventually recovered and 3 died. 2 of the deaths were attributed to intercurrent infection and one to uncontrollable bleeding.

Repeated transfusions of whole blood were necessary to tide the patient over the acute phase of the disease. No other therapeutic measure afforded relief from the manifestations of bone marrow depression. Figure 2 is a reproduction of the course of Case 1. The various measures and their hematologic effects are graphically portrayed. None of the therapeutic agents seemed to influence the course of the illness. Whole blood administration, when effective, was only transiently palliative.

#### COMMENT

The mechanism of bone marrow depression by atabrine is complicated by the demonstration that atabrine may remain in body tissues after the drug has been discontinued.<sup>8</sup> Consequently, two factors may cause development of the hypoplastic marrow due to atabrine: (1) the initial cumulative depression due to ingestion of the drug and (2) the perpetuation of the depression by residual stores of drug within the body. Recovery is spontaneous and gradual, apparently uninfluenced by medication.

#### SUMMARY

Seven patients with severe hypoplastic anemia were studied at an army Tropical Disease Center. Four of the 7 patients had concomitant dermatitis. The relationship of the prolonged administration of atabrine to the anemia and dermatitis is presented. A hematologic remission could not be induced by specific therapeutic measures. Four of the 7 cases recovered spontaneously.

#### ACKNOWLEDGMENT

The authors are indebted to Miss Mary L. Boyd for her painstaking supervision of the hematologic studies.

#### REFERENCES

- <sup>1</sup> NOOGIN R. D. AND CALLAWAY J. L. Generalized exfoliative erythroderma following atabrine. North Carolina M. J. 3: 139, 1942.
- <sup>2</sup> SCHMITT C. L., ALPINS O. AND CHAMPERS G. A clinical investigation of a new skin entity. Arch. Derm. & Syph. 55: 226, 1945.
- <sup>3</sup> NISBET I. W. A new cutaneous syndrome occurring in New Guinea and adjacent islands. Arch. Derm. & Syph. 52: 221, 1945.
- <sup>4</sup> LIVINGOOD C. S. AND DIEUAIDE F. R. Atypical lichen planus. J. A. M. A. 129: 1091, 1945.
- <sup>5</sup> GOLDBERG L. C. An unusual lichenoid dermatosis. J. A. M. A. 130: 775, 1946.
- <sup>6</sup> BAZEMORE J., JOHNSON H., SWANSON E. AND HATMAN J. Relation of quinacrine hydrochloride to lichenoid dermatitis (atypical lichen planus). Arch. Derm. & Syph. 54: 308, 1946.
- <sup>7</sup> CUSTER R. P. Aplastic anemia in soldiers treated with atabrine (quinacrine). Am. J. M. Sc. 212: 211, 1946.
- <sup>8</sup> Malaria Research Unit, 3rd Medical Laboratory. Report No. 13. Clinical and laboratory studies on atypical lichen planus with particular reference to the role of atabrine. Dec. 3, 1944.
- <sup>9</sup> PARKER L. G. Blood and bone marrow concentration of atabrine and its role in aplastic anemia. J. Lab. & Clin. Med. 33: 827, 1948.

# INFLUENCE OF FIBRINOGEN CONCENTRATION UPON PLASMA PROTHROMBIN TIME

By MURRAY WEINER, M D , AND SHEPARD SHAPIRO, M D

ESTIMATION of the prothrombin time of whole and diluted (12.5 per cent) plasma has been shown to have advantages over the usual one-stage procedure in which whole plasma alone is employed.<sup>1a, b</sup> Recently, Deutsch and Gerarde indicated that the prothrombin time of diluted plasma may be influenced by variations in the fibrinogen level.<sup>2</sup> Their study was on rabbit plasma. In the present communication, data are given concerning the fibrinogen concentration in human plasma in the presence of changes in diluted (12.5 per cent) plasma prothrombin time.

The following studies were made: Simultaneous estimation of prothrombin time and fibrinogen concentration of plasma in (1) normal subjects, (2) cases of hyperprothrombinemia,<sup>3, 4</sup> (3) cases of hypoprothrombinemia.

## METHODS

Estimation of the prothrombin time was made by the procedure previously described.<sup>1a</sup> Fibrinogen concentration was established by determination of the nitrogen content of plasma before and after the contained fibrinogen was coagulated and removed according to the method described by Peters and Van Slyke, modified for micro-kjeldahl technique.<sup>13</sup>

## RESULTS

In the tables given below, the prothrombin time of the diluted (12.5 per cent) plasma is given in seconds. The normal standard is 39.5 seconds, standard deviation  $\pm 2.5$ . (The whole plasma prothrombin time plays no part in the present discussion.) The fibrinogen values are given in milligrams per 100 ml.

Figure 1 is a scatter diagram of 76 simultaneous determinations of plasma fibrinogen and diluted plasma (12.5 per cent) prothrombin time. There is no correlation between the two.

Table 1. In 4 normal persons, simultaneous prothrombin time and fibrinogen estimations were made. The prothrombin time was normal in each case. The fibrinogen values also were normal. In 3 cases of spontaneous hyperprothrombinemia, the fibrinogen results were within the normal range.

Table 2. Normal persons and cases of liver disease were given large doses of synthetic vitamin K intravenously. Each type of response is represented. Prothrombin time unchanged, reduced, or increased. All of the fibrinogen values were within normal limits and no parallelism was observed between the shift in the prothrombin time and the fibrinogen levels.

From the Third (New York University) Division, Goldwater Memorial Hospital, Welfare Island, N. Y., and the Department of Medicine, New York University College of Medicine.

Aided by a grant from the Blood Transfusion Association of Greater New York.

## DISCUSSION

The study was made in normal, hyperprothrombinemic (both spontaneous and induced) and hypoprothrombinemic bloods. Correlation between fibrinogen concentration and variations in prothrombin time is strikingly lacking. All the fibrinogen values are within the normal range while the prothrombin times extend

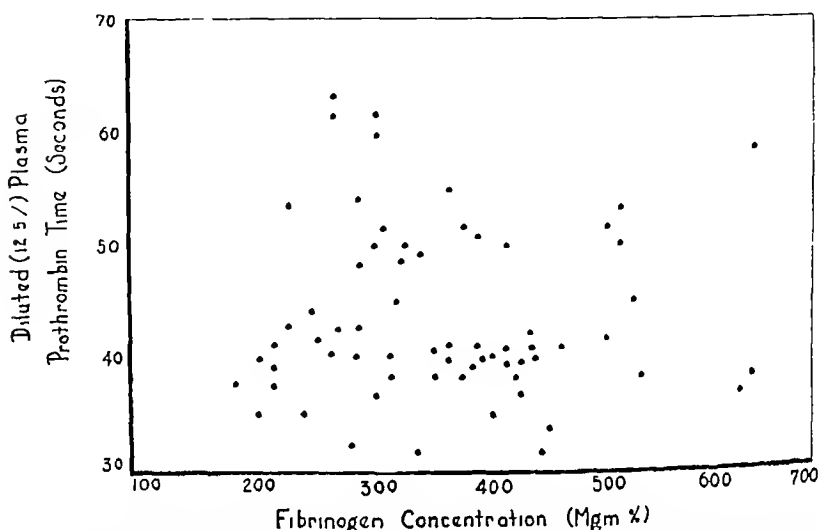


FIG. 1—Scatter graph representing the fibrinogen concentration and diluted (12.5%) plasma prothrombin time of 76 samples of blood. The series includes cases in which the prothrombin time is normal, prolonged (hypoprothrombinemia) and reduced (hyperprothrombinemia).

TABLE 1—Simultaneous Determinations of Plasma Fibrinogen and Diluted (12.5%) Plasma Prothrombin Time in 4 Normal Subjects and in 3 Cases of Hyperprothrombinemia<sup>2</sup>

Case	Prothrombin Time	Fibrinogen
	sec	mgm per 100 ml
A. Normal subject	42.4	410
B. Normal subject	40.0	310
C. Normal subject	37.8	420
D. Normal subject	39.8	205
E. Spontaneous hyperprothrombinemia	32.8	450
F. Spontaneous hyperprothrombinemia	32.8	330
G. Spontaneous hyperprothrombinemia	35.4	430

throughout the gamut of normal, increased and reduced activity. Especially instructive is the contrast between Case H and Case J. In the former, the serial prothrombin time estimations remained within the normal range despite an increase in fibrinogen content from 246 to 609 mg per 100 ml plasma. In Case J, a significant prolongation of prothrombin time occurred simultaneously with elevations in

fibrinogen values from 240 (at which time, the prothrombin time was normal) to 410 mg, when it was definitely at a hypoprothrombinemia level. Case I is likewise noteworthy, because in it, the fibrinogen concentration showed practically no

TABLE 2.—*Simultaneous Determinations of Plasma Fibrinogen and Diluted (1:2.5%) Prothrombin Time after Vitamin K*

Description of Cases	Days	Prothrombin Time	Fibrinogen mgm per 100 ml
		sec	
Case H Normal subject Prothrombin time unchanged after vitamin K. Variations in fibrinogen values marked	1	40.8	311
	2	44.0	
	3	41.8	246
	4	39.4	609
Case I Normal subject Prothrombin time reduced following vitamin K. Fibrinogen values relatively constant	1	42.4	410
	2		
	3	35.2	400
	4		
	5	31.8	450
Case J Hepatic cirrhosis Positive vitamin K tolerance test <sup>*</sup> Prothrombin time increased following vitamin K. Fibrinogen values increased when prothrombin time prolonged	1	45.8	210
	2	40.4	240
	3	41.9	360
	4	48.0	340
	5	52.0	410
Case K Hepatic cirrhosis Prothrombin time initially prolonged and temporarily reduced following vitamin K. Fibrinogen values not increased when prothrombin time reduced	1	53.6	500
	2	34.8	450
	3	41.8	400
	4	52.0	510
Case L Hepatic cirrhosis. Positive vitamin K tolerance test Fibrinogen concentration not reduced on day prothrombin time prolonged	1	49.0	260
	2	42.4	240
	3	41.0	205
	4	64.9	250
Case M Hepatic cirrhosis No correlation of increased prothrombin time with reduced fibrinogen concentration	1	42.4	320
	2	52.4	380
	3	46.2	300
	4	54.2	370
	5	49.6	240

\* Seventy six mg of synthetic vitamin K (2-methyl-1,4-naphthohydroquinone diphosphoric acid ester tetrasodium salt [Synkayvite]) was given intravenously on each of the first four days. Blood for prothrombin time and fibrinogen estimation was withdrawn each day before administration of the daily dose of vitamin K.

alteration at the time the prothrombin time became reduced to the hyperprothrombinemia range.

Deutsch and Gerarde,<sup>2</sup> working with rabbit plasma, induced in vitro a reduction of the prothrombin time of 10 per cent plasma from 33 seconds to 22 seconds by

adding 150 mg of beef fibrinogen per 100 ml plasma. They pointed out that there was considerable species variation in this effect. Our findings indicate that the results obtained with rabbit plasma are not applicable to man. The normal range of fibrinogen content of the human plasma is 200–600 mg per 100 ml. If fibrinogen variations had as great an effect on human diluted plasma as is implied by the data on rabbit plasma referred to above, it is difficult to understand how the mean prothrombin time of the diluted (12.5 per cent) plasma of 39.5 seconds, obtained by studying blood from several hundred normal subjects, could have a standard deviation of only  $\pm 2.5$ . This fact substantiates further the belief that the usual fibrinogen range of diluted (12.5 per cent) plasma in man (30 mg to 75 mg per 100 ml) does not alter significantly the accelerated clotting time.

Data offered by Owren<sup>7</sup> in his extensive study of the coagulation mechanism support the above conclusion. Owren demonstrated that the critical low level of fibrinogen below which clotting time increases sharply, varies with the prothrombin concentration. In 10 per cent plasma, the fibrinogen concentration must be reduced to below 20 mg per 100 ml before a significant effect upon accelerated clotting time is noted. It is at this dilution that the quality of the clot becomes poor and difficult to detect. The 12.5 per cent plasma yields a firm and easily discernible clot. The 8 per cent plasma often gives a rather poor clot. The range between these two dilutions, 12.5 per cent and 8 per cent, includes the critical fibrinogen concentration below which clotting time rises sharply. This is consistent with our experience of two instances in which 12.5 per cent plasma yielded poor clots and the fibrinogen concentrations were 100 mg per cent and 120 mg per cent. Thus, only in these very rare cases of hypofibrinogenemia (below 150 mg per cent) does fibrinogen concentration become a factor in 12.5 per cent plasma prothrombin time determination. It appears that a poor clot, which is a rare occurrence, may be considered as a warning that the fibrinogen level is sufficiently low to cause an alteration in the accelerated clotting (prothrombin) time.

An obvious modification of the technic would be to use fibrinogen solution as diluent in place of normal isotonic saline. This has been done by Thordarson<sup>8</sup> and by Link and his students.<sup>9</sup> In man, it has been our experience that the prothrombin time of diluted (12.5 per cent) plasma may be affected in an unpredictable fashion thereby. In some instances, the prothrombin time remained unaltered, while in others it became extended. When the protein solution is used as diluent additional factors such as questionable purity and stability may be introduced. These additional variable factors do not exist if saline is used as diluent.

The data presented show considerable difference in fibrinogen concentration with no corresponding variations in prothrombin time. Reliable estimations of the diluted (12.5 per cent) plasma prothrombin time can be made at the low normal level of fibrinogen (180 mg per 100 ml) as well as at the high level of 650 mg per 100 ml.

It has been found by Foster and Whipple,<sup>10</sup> and later emphasized by Link,<sup>9</sup> that fibrinogen is a very labile plasma protein and fluctuates readily in response to a variety of factors. It is important to point out that massive doses of dicumarol may depress the fibrinogen level of plasma<sup>11</sup> but that at therapeutic dosage levels of

dicumarol the fibrinogen concentration is maintained within the normal range. This has been demonstrated in animals<sup>9</sup> and in man.<sup>12</sup>

### SUMMARY

The effect of the normal variations of fibrinogen concentration (180 mg per cent to 650 mg per cent) on the diluted (12.5 per cent) plasma prothrombin time in man, as observed in this study, is not significant.

### REFERENCES

- <sup>1</sup> (a) SHAPIRO S, SHERWIN S, REDISH M AND CAMPBELL H A. Prothrombin estimation: A procedure and clinical interpretations. *Proc Soc Exper Biol & Med* 50: 85, 1942.
- (b) LINK K P. The anticoagulant from spoiled sweet clover hay. *Harvey Lecture Series* 38: 1943-4, 162-216.
- <sup>2</sup> DEUTSCH H F AND GERARDE H W. Biophysical studies of blood plasma proteins. Effect of fibrinogen on prothrombin time. *J Biol Chem* 166: 381, 1946.
- <sup>3</sup> SHAPIRO S. Hyperprothrombinemia: A premonitory sign of thromboembolization (description of a method). *Exper Med & Surg* 2: 103, 1944.
- <sup>4</sup> —, AND RICHARDS, R K. The prothrombin response to large doses of synthetic vitamin K in liver disease. *Ann Int Med* 22: 841, 1945.
- <sup>5</sup> UNOER, P AND SHAPIRO S. Hyperprothrombinemia induced by vitamin K in human subjects with normal liver function. *Blood* 3: 137, 1948.
- <sup>6</sup> — AND —. The prothrombin response to the parenteral administration of large doses of vitamin K in subjects with normal liver function and in cases of liver disease: A standardized test for the estimation of hepatic function. *J Clin Investigation* 27: 39, 1948.
- <sup>7</sup> OWREN P A. The coagulation of blood. Investigations on a new clotting factor. *Acta Med Scand Suppl* 194: 128, 1947.
- <sup>8</sup> THORDARSON A M. Ein Modifikation von Prothrombinbestimmung. *Nord Med* 19: 331, 1943. Quoted by JANSEN K F. *Dikumarin*. Ejnar Munksgaard, 1944.
- <sup>9</sup> LINK, K P. The anticoagulant 3,3'-methylenebis (4-hydroxycoumarin). *Fed Proc* 4: 176, 1945.
- <sup>10</sup> FOSTER, D P AND WHIPPLE G H. Blood fibrin studies. Part IV. Fibrin values influenced by cell injury, inflammation, intoxication, liver injury and the Eck fistula. *Am J Physiol* 58: 407, 1921.
- <sup>11</sup> IRISH U D AND JAMES L B. The effect of dicumarol upon plasma fibrinogen. *Am J Physiol* 143: 101, 1945.
- <sup>12</sup> Unpublished Data.
- <sup>13</sup> PETERS, S AND VAN SLYKE, D. *Quantitative Clinical Chemistry*, vol. 1. Baltimore: Williams & Wilkins, 1932.



# ABSTRACTS

JOSEPH F ROSS M D *Editor*

ABSTRACTERS

HELEN W BELDING M D, Winston Salem N C.  
SHEILA T CALLENDER M D Oxford England  
GEORGE E CARTWRIGHT M D, Salt Lake City  
ROGER C CRAFTS PH D Boston  
CHARLES P EMERSON M.D Boston  
SOLOMON ESTREN M D New York  
CLEMENT A FINCH M D Seattle

OLIVER P JONES PH D Buffalo  
CONRAD MAIER M D Zurich, Switzerland  
MILOS NETOUSEK, M D Prague Czechoslovakia  
JEAN P SOULIER M D Paris  
RAMON M. SOAREZ M.D San Juan, Puerto Rico  
WM N VALENTINE M.D, Pacific Palisades Cal  
JAN WALDENSTRÖM M D Upsala Sweden

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## BLOOD COAGULATION AND HEMORRHAGIC DISEASE

CIRCULATING ANTICOAGULANTS A TECHNIQUE FOR THEIR DETECTION AND CLINICAL STUDIES C. L. Gailly  
R C Hartmann and W I Morse II From the Clinical Microscopy Division, Department of Medicine  
The Johns Hopkins University and Hospital Baltimore, Md Bull Johns Hopkins Hosp. 54  
255-268 1949

A test for circulating anticoagulants is described utilizing the effect of platelet free plasma on normal blood. The preparation of the platelet free plasma depends on scrupulous technic: siliconed syringes, test tubes and pipets; handling the blood at low temperatures and two separations by centrifugation at 7000 and 12 000-14 000 rpm. By this method amounts of added heparin as low as 0.001 mg per ml platelet free plasma were detectable. In clinical studies eight instances of a circulating anticoagulant were detected. In only one did the addition of toluidine blue suggest the presence of a heparin-like substance. In 9 cases of thrombocytopenia the anticoagulant assays were negative. These interesting studies point up the probability that circulating anticoagulants are probably present much more commonly than suspected in the past and the suggested technic offers another approach to the study of hemorrhagic diatheses. From the standpoint of widespread use the meticulous technic necessary for the successful preparation of platelet free plasma unfortunately is a limitation on its general availability.  
W N V

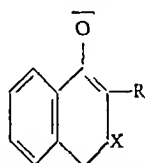
HUMAN PROTHROMBIN QUANTITATIVE STUDIES ON THE PLASMA LABILE FACTOR AND THE RESTORATIVE EFFECTS OF NORMAL HYPOFIBRINOGENEMIC AND HEMOPHILIC PLASMA ON THE PROTHROMBIN OF STORED PLASMA B Alexander and A de Vries From the Medical Research Laboratory Beth Israel Hospital and the Department of Medicine Harvard Medical School Boston Mass J Clin Investigation 21  
24-31 1949

The conclusions of Loomis and Seegers (Am J Physiol 148 563 1947) namely that deterioration of fibrinogen accounts for lengthening of the prothrombin time in stored plasma and that reactive fibrinogen is necessary for prothrombin activity measured by the one stage technic have been tested by the authors of this report employing as a reagent afibrinogenemic plasma from a subject with spontaneous fibrinopenia. Inasmuch as the retarded prothrombin time of stored plasma was fully restored upon the addition of this plasma it is concluded that fibrinogen is not the factor whose deterioration is responsible for the alteration in the clotting properties of stored blood a conclusion that is further substantiated by the restorative properties of normal plasma rendered fibrinogen free by means of thrombin. This factor (labile factor) which is also present in  $\text{BaSO}_4$  plasma (prothrombin free) as well as in hemophilic plasma is required in adequate amount for normal prothrombin activity. It is pointed out that the use of prothrombin free ( $\text{BaSO}_4$ -treated) plasma containing not only the labile factor but also fibrinogen and antihemophilic activity in normal amounts is preferable to saline as a diluent in the performance of the one-stage prothrombin test since a reduction of the concentration of these important non prothrombin constituents is thereby avoided.

C. P. E.

LES DIVERS GROUPES DE SUBSTANCES SYNTHÉTIQUES DOUÉS D'UNE ACTIVITÉ ANTIVITAMINIQUE K ET LA SIGNIFICATION BIOLOGIQUE DES RÉSULTATS OBTENUS (DIFFERENT COMPOUNDS WITH ANTI K ACTIVITY AND THE SIGNIFICANCE OF THE RESULTS OBTAINED) *C. Mentzer* Laboratoire de Chimie Biologique Faculté des Sciences Lyon Bull Soc Chim Biol 30 872-884 1948

A study was made by Mentzer to determine the relation between the chemical structure of various compounds similar to dicoumarol and their anticoagulant activity. His conclusions are similar to those of Link and his collaborators, but whereas the American authors believe that the B cycle is necessarily an hydroxy 4 pyrone 1,2. Mentzer estimates that other cycles such as thiopyrone, quinon, pyridin, cyclo-pentanedione are able to coöper to the molecule the same activity as the B pyronic cycle. In conclusion all the active compounds have the same structure which can be schematized as follows:



$X = (\text{CO}-\text{O}) \text{ or } (\text{CO}-\text{NH}) \text{ or } (\text{CO}) \text{ or } (\text{S})$

If R is an atom of chloride or of hydrogen or a complex of at least 6 carbon atoms the molecule behaves as an antivitamin K. All the compounds also have in common the O atom in B; this O atom can belong to an enolic or ketonic group but the blockage of this oxyhydride function suppresses the activity.

J P S

ACQUIRED AFIBRINOGENEMIA IN PREGNANCY *W C Maloney, W J Egan and A J Gorman* From the Medical and Obstetrical Services, St Elizabeth's Hospital, Boston, Mass. New England J Med 240 596-598 1949

This interesting case report describes a hemorrhagic diathesis in a pregnant woman at term, characterized by a critical decrease in fibrinogen. By use of blood transfusions and fraction I of Cohn it was possible to remove a dead fetus surgically after which rapid recovery of the mother occurred. The authors discuss the possible role of fibrinolysis in this type of acquired afibrinogenemia.

C A F

FIBRINOLYSIS AND THE FLUIDITY OF THE BLOOD POST MORTEM *R H Mole* From the Radcliffe Infirmary, Oxford England J Path & Bact 60 413-427 1948

The finding of fluid and incongluable blood at autopsy is not an uncommon occurrence. However, the explanation of this phenomenon has not been subject to critical investigation and it is for this reason that the present study was undertaken. Blood was obtained from the heart and great vessels of 61 cadavers at routine but not consecutive autopsies. Observations on the fibrinolysis in supernatant serum were made by using a modification of Macfarlane's method (1937). A regional difference in the rate of spontaneous intravascular coagulation as well as in fibrinolytic activity was found. Cadaver fibrinolysis is nondialyzable, precipitated at neutral pH in 50 per cent saturation of ammonium sulphate and its activity is destroyed by pepsin. It appears to be a globulin. The appearance of fibrinolysis seems to be part of the body's general reaction to injury and it is probably produced by endothelium.

O P J

CAUSE AND SIGNIFICANCE OF SEASONAL VARIATIONS IN THE HAEMORRHOIC TENDENCY IN THE NEWBORN *E Kerpel-Fronius, F Varga and E Kutas-Pal* From the University's Children's Clinic, Pecs, Hungary Arch Dis Childhood 23 87-89 1948

In a study of 10,000 newborn children over a period of five years, the authors found a seasonal variation in the incidence of melena, cerebral hemorrhage, and cephalhematoma. The peak period was identical for each and occurred during winter and spring, with diminution during summer and autumn. Since the trauma of delivery presumably did not have a seasonal variation, an explanation for the incidence variations was sought in possible changes in the clotting mechanisms and in the capillary fragility.

The most marked prothrombin reductions, the authors state, have been noted (in the literature) to occur in the winter and spring. No other defects of clotting mechanism were known to be of seasonal incidence.

The authors tested capillary fragility in 233 healthy children at various times of the year using both positive and negative pressure methods, and found that the incidence of increased capillary fragility increased in winter and spring, diminished in summer and was minimal in late summer. In addition they noted that conjunctival petechiae in the newborn which are supposedly due to rupture of capillaries during labor were most numerous in spring and winter. There was thus a distinct parallelism in incidence of cerebral hemorrhage, cephalhematoma, conjunctival petechiae and excessive capillary fragility.

The authors suggest that the use of vitamins K and P during the latter months of pregnancy may prevent these hemorrhagic tendencies.

S. E.

**HEMOPHILIA. PROBLEM OF SURGICAL INTERVENTION FOR ACCOMPANYING DISEASES. REVIEW OF THE LITERATURE AND REPORT OF A CASE.** C. G. Craddock, L. O. Fenninger and B. Simmons. From the University of Rochester School of Medicine and Dentistry and the Departments of Medicine and Surgery of Strong Memorial and the Rochester Municipal Hospital, Rochester, N. Y. *Ann Surg* 128: 888-903, 1948.

A case of hemophilia with the complication of acute appendicitis is presented. The patient received intensive antihemorrhagic therapy but despite a normal clotting time he continued to bleed profusely and expired four days postoperatively. A discussion of the significance of continued hemorrhage in the presence of a normal *in vitro* clotting time and its relation to the fundamental defect in hemophilia is presented. The authors conclude that the mere deficiency of the substance, antihemophilic globulin, cannot be the sole abnormality of coagulation in hemophilia. Emphasis is placed on the failure of the coagulation time to indicate the severity of the hemorrhagic tendency or the degree of response to treatment, the difficulty in choosing a suitable case for operation and the great difference in controlling interval hemorrhage as opposed to bleeding from an external site.

The authors reviewed the literature for instances of internal operative procedures in patients with hemophilia. Of four previously reported cases in whom the diagnosis of hemophilia was unequivocal two died from hemorrhage following operation while two recovered.

G. E. C.

**ACTION OF INTRAVENOUS INJECTIONS OF HISTAMINE ON THE BLOOD OF HEMOPHILIC CHILDREN.** H. N. Sanford, S. Butler and S. R. Kennedy, Jr. From the Presbyterian Hospital and the Department of Pediatrics, Rush Presbyterian Division of the University of Illinois, Chicago, Ill. *Am J Dis Child* 76: 609-615, 1948.

Following the observation of a slight decrease in blood coagulation time in adults with migraine treated with intravenous histamine, 6 hemophilic children were given histamine injections in increasing amounts (usually 0.3, 0.6, 0.8 and 1.0 mg.) on successive days during active cycles of bleeding. Determinations of the coagulation time were made on whole blood, plasma and platelet free (?) plasma obtained by centrifugation in waxed chilled tubes. A greater and more prolonged, although not permanent, decrease in coagulation time occurred in the hemophilic group with cessation of bleeding. The total number of platelets was not affected.

The authors conclude that this decrease in coagulation time is due to increased platelet disintegration (possibly of new and more normal platelets), their sole premise being that the defect in hemophilia is the result of abnormal qualitative platelet function. Although it has been shown that platelets are also essential for normal thromboplastic activity, the role of a plasma factor in hemophilia is not considered. This report is of interest, however, and further clinical trial and investigation with histamine in hemophiliacs appears warranted and may possibly reveal the mechanism of action which at present is not clear.

H. B. M.

**THE ROLE OF PLATELETS IN THE COAGULATION OF THE BLOOD.** A. J. Quick, J. N. Shanberg and M. Sefourni. From the Department of Biochemistry, Marquette University School of Medicine, Milwaukee, Wis. *Consilium* *Am J M Sc* 217: 198-205, 1949.

A technic was devised for varying the number of platelets without otherwise altering the plasma. The effect of the number of platelets was studied and the following observations made: (1) The greater the number of platelets, the sooner clot retraction begins and the smaller the final clot; (2) clot retraction

tion is characterized by a relatively long latent period followed by an accelerated phase and protracted completion (3) within a wide range in the number of platelets no significant change in coagulation time can be observed (4) as the number of platelets is diminished the speed of prothrombin consumption is decreased but within normal limits the final amount of prothrombin converted approximates a fixed value (5) below a critical number of platelets the consumption of prothrombin stops after a relatively short time This suggests that plasma contains an agent that inactivated the platelet enzyme, (6) in thrombocytopenic purpura of sufficient severity the consumption of prothrombin may be markedly diminished This suggests that in thrombocytopenic purpura a serious defect in coagulation is present which has heretofore been unrecognized because it is masked by a normal coagulation time

G E C

ÉTUDE DES MÉGAKARYOCYTES ET DES PLAQUETTES DANS DIVERS SYNDROMES HÉMORRAGIQUES (STUDY OF MEGAKARYOCYTES AND PLATELETS IN VARIOUS HEMORRHAGIC SYNDROMES) L. Revol and P. Morel  
Laboratoire de Pathologie Interne et Service du Pr. Croizat Lyon Sang 20 23-59 1949

The authors discuss the normal features of megakaryocytes in marrow smears They found in normal marrow more megakaryocytes than are usually stated to be present (up to 2,000 per million nucleated cells) but they agree about the percentage of the different cells from the megakaryoblast to the old cells They study platelet formation after splenectomy and find that platelets are essentially produced by the cytoplasm but sometimes a fragmentation of the nucleus is observed In 6 cases of idiopathic thrombopenias they found what Revol himself described in 1939 and what was confirmed by several authors, that is an increase of megakaryocytes (above 1,600 for 1 million of nucleated cells) but without increase of the young forms as it is commonly said

After splenectomy they found in 4 cases a striking platelet formation in megakaryocytes which was to be found as soon as one and a half hours after the operation with a maximum at the third day At the same time the number of megakaryocytes was reduced (from 5,900 to 3,900 in the average) In 2 cases however in spite of the same initial bone marrow splenectomy was not followed by the same platelet formation and the thrombocytopenia was not cured

In 2 cases of acquired thrombocytopenia the megakaryocytes were numerous in the marrow smears after splenectomy in the first case and transfusions in the second case a very slow platelet formation was observed In 3 cases of infectious thrombocytopenia the megakaryocytes were plentiful but there were numerous cytologic alterations

In 6 cases of hemorrhagic syndromes without thrombocytopenia they usually found an active bone marrow rich in megakaryocytes They discuss the effect of splenectomy and the advisability of this procedure

Twenty-seven good microphotographs illustrate this interesting study which ends with conclusions about the indications of splenectomy A very great number of megakaryocytes the lack of platelet formations seem to indicate the splenectomy

J P S

GAUCHER'S DISEASE WITH THROMBOCYTOPENIA AN INSTANCE OF SELECTIVE HYPERSPLENISM A CASE REPORT  
F. W. Davis, Abraham Genecin and Ernest W. Smith From the Medical Clinic The Johns Hopkins Hospital Bull. Johns Hopkins Hosp. 84 176-179 1949

The authors report an instance of thrombocytopenia unassociated with anemia or leukopenia in which hypersplenism secondary to Gaucher's disease was apparently the etiology Splenectomy resulted in correction of the thrombopenia and the hemorrhagic diathesis

W N V

THE INFLUENCE OF BRIEF PERIODS OF STRENUOUS EXERCISE ON THE BLOOD PLATELET COUNT E. B. Gribbin and A. T. Miller Jr From the Laboratory of Applied Physiology and Department of Physiology University of North Carolina School of Medicine Chapel Hill N. C. Science 109 64 1949

Reports in the literature on the effects of exercise on the platelet count are in conflict This work was performed in an attempt to solve whether exercise actually does change the platelet count Exercise consisted of running on a treadmill for five minutes at a speed of 7 miles an hour and a grade of 17.5 per cent or for two minutes at 12 miles an hour at zero grade Blood was obtained before exercise immediately after exercise and 10, 30, 60 and 90 minutes after exercise In spite of the fact that there was a

60-100 per cent increase in the leukocyte count, there was no increase in the platelet count. The authors suggest that the increased velocity of circulation may have destroyed the very labile platelets which may have covered up any increase in platelets

R C C

ROLE OF SPLENECTOMY IN THROMBOGENIC PURPURA *G Bagardus, J G Allen, L O Jackson and C L Spurr*  
From the Departments of Surgery and Medicine University of Chicago Chicago Ill Arch Surg  
58 16-27 1949

The authors present data on 20 cases of thrombogenic purpura 20 of which were treated medically and 10 treated by splenectomy Five recurrences were noted in the splenectomized group and 2 in the medically treated group Three of the patients with recurrence had the acute form of the disease and two the chronic The pathogenesis of the syndrome is discussed with emphasis on the importance of the capillary factor and the possible role of the entire reticuloendothelial system

The results are rendered somewhat difficult to evaluate by the fact that 14 of the patients were under the age of 22 years and 11 under 10 years of age and because 6 of the medically treated group and none of the surgically treated group had had symptoms for less than four weeks

W N V

THE EFFECT OF RUTIN IN THE CONTROL OF BLEEDING INTO THE RETINA *R W Hollenhorst and H P Wagner*  
From the Section on Ophthalmology Mayo Clinic, Rochester Minnesota Am J M. Sc 27 227-231, 1949

The clinical literature of the use of rutin in conditions of increased capillary fragility is discussed. The article serves as a useful review of the subject but the authors justifiably stress the variability of reports and their inability to draw any definite conclusions with the evidence at hand

C A F

EVALUATION OF THE VARIOUS CLINICAL SIGNS OF THROMBOPHLEBITIS AND EXPERIENCE IN THERAPY WITH ANTICOAGULANTS *D A Felder* From the Department of Surgery University of Minnesota, Minneapolis, Minn Surg Gynec & Obst 88 337-350 1949

The results of treatment of 91 cases representing 105 extremities with deep thrombophlebitis are reported together with a detailed discussion of the diagnostic signs methods of treatment type of venous thrombosis, and the primary disease process For practical purposes both the bland and inflammatory thromboses were called thrombophlebitis With the exception of eight patients who were treated with vein ligation anticoagulants (dicumarol and heparin) were used with an average of ten days of bed rest in, unless contraindicated mild Trendelenburg position

Although most of the patients had had one pulmonary embolism at the time of diagnosis of thrombophlebitis the results of anticoagulant therapy were considered satisfactory in that the incidence of secondary embolism was reduced from an expected 30 per cent to 2.17 per cent and that of secondary fatal embolism from an expected 25 per cent to zero An analysis of the primary condition indicated the importance of prophylactic postoperative anticoagulant therapy in patients with cancer and in those undergoing major gastrointestinal surgery hysterectomies and hip fixations

The controversy as to the relative merits of anticoagulant therapy vs vein ligation in the prevention and treatment of thromboembolism will probably remain unsettled for some time It would seem however that both have a place in the management of this disorder and that the indications for each should be determined more on the basis of the type of thrombophlebitis the underlying disease and condition of the patient availability of laboratory control and the estimated risk of fatal embolus It is quite possible that the incidence of death from pulmonary embolism is much lower than generally believed (see Surg Gynec & Obst 88 373 1949)

H B M

## TRANSFUSION

ON THE CHEMICAL STERILIZATION OF BLOOD AND BLOOD PLASMA *F W Hartman G H Mangen N Friley and E Jackson* From the Department of Laboratories Henry Ford Hospital, Detroit Michigan Proc. Soc. Exper Biol & Med 78 148-154 1949

In the hope that a means might be supplied for reducing the high incidence (4.5-7.2 per cent) of homologous serum jaundice or of eliminating this risk altogether in transfusion recipients and in view of the essential unavailability of effective irradiation techniques for the sterilization of blood and blood derivatives the authors have investigated the merits of nitrogen mustard (HN) as a sterilizing agent. Selection of this compound for study was based on the following considerations: its presumed effect on nucleoproteins; its ready susceptibility to spontaneous hydrolysis in buffered aqueous solution; forming relatively nontoxic end products; the parallelism of its activity with that of ionizing radiations; and its availability in purified form suitable for parenteral administration.

It was demonstrated that HN is capable of effective bactericidal and virucidal action in whole blood, blood plasma and blood serum without causing major alterations in the properties of either the plasma components or the red blood cells. Virucidal potency appeared to be greatly enhanced by decreasing the pH to 7.2 or below, possibly attributable to a reduction in the rate of HN decomposition or to a lessened reactivity with other competing substances and at these pH levels sterilization was considered to be accomplished with concentrations of the drug not exceeding 500 mg per liter. No evidence of antigenic or other toxic reactions was produced in two dogs and two humans receiving repeated injections of plasma so treated. Plasma complement, immune bodies, phosphatase and fibrinogen were apparently unaffected by exposure to sterilizing doses, but a marked reduction of prothrombin activity was observed. In vitro studies failed to demonstrate a significant increase in the rate of erythrocyte deterioration in stored blood following the application of virucidal dosages of HN. In vivo erythrocyte survival studies, however, have not as yet been completed.

C P E

## IRON THERAPY AND METABOLISM

PREPARATION AND STANDARDISATION OF SACCHARATED IRON OXIDE FOR INTRAVENOUS ADMINISTRATION

J. A. Nissem and J. M. Robson. From Guy's Hospital Medical School, University of London, London, England. *Lancet* 1: 686-689, 1949.

Details of methods of preparation and toxicity tests on various samples of saccharated oxide of iron are described. The toxicity varied considerably and seemed to be the result of gradual precipitation of free iron. This in turn it was thought might depend on the rate of metabolism of the sugar part of the molecule. Mice given lethal doses showed hemorrhagic lesions, probably due to multiple capillary emboli from iron precipitation. As a consequence of the difficulties encountered in producing uniform preparations the authors suggest that biologic standardization is essential.

S C

THE DERMAL EXCRETION UNDER CONTROLLED ENVIRONMENTAL CONDITIONS OF NITROGEN AND MINERALS

IN HUMAN SUBJECTS WITH PARTICULAR REFERENCE TO CALCIUM AND IRON. H. H. Mitchell and T. S. Hamilton. From the Division of Animal Nutrition, University of Illinois, Urbana, Ill. *J. Biol. Chem.* 178: 345-361, 1949.

In studies of 6 subjects exposed to humid heat, these authors found iron in amounts of 1 to 3 mg/liter of sweat. They estimated a daily loss of about 6.5 mg iron under minimal sweating conditions.

This magnitude of iron excretion is not in harmony with present concepts of iron metabolism. In fact, such a daily loss would appear to be more than the normal individual is able to absorb. Obviously further studies are necessary before these findings can be interpreted.

C A F

## PIGMENT METABOLISM

INFLUENCE OF FOLIC ACID ON PORPHYRIA. V. Avasniškova. From the 3rd Medical Clinic, Charles University, Prague. *Čes. lek. čas.* 8: 633, 1948.

A woman suffering from a cutaneous form of porphyria was eliminating 200 gamma uroporphyrin (and the same amount of coproporphyrin III) per liter of urine. Porphyria was accompanied by severe hypochromic anemia and raised level of plasma iron. Folic acid administered in the daily dose of 15 mg proved to be highly effective. Skin manifestations disappeared, pigmentations cleared up, general feeling improved and uroporphyrin disappeared entirely from the urine.

M. N

CLINICAL INVESTIGATIONS IN THE DIFFERENTIATION OF STERCOBILINE AND UROBILINE WITH THE PENTDYOPENT REACTION *W. Stieh* From I. Medizin. Klinik der Universität München (Germany) *Klin. Wochschr.* 365-367, 1948

Differentiation of stercobiline and urobiline is made possible by the pentdyopent reaction. Clinical examinations showed that the hitherto assumed urobilinuria is actually a stercobilinuria. Presence of urobiline IX<sub>a</sub> in urine is always sign of a pathologic process. Stercobilinuria and elimination of stercobiline in the feces is found in pernicious anemia, hemolytic jaundice, malaria and in parenchymatous icterus. Pure urobilinuria can be encountered in icterus of total biliary occlusion. Preponderant urobilinuria can be seen in beginning parenchymatous liver affection. Increase of stercobiline comes later. The choical differentiation of both substances opens new diagnostic possibilities.

C. M.

MECHANISM OF HYPERBILIRUBINEMIA IN THE NEWBORN INFANT. EXPERIMENTAL DEMONSTRATION OF FUNCTIONAL HEPATIC IMMATUREITY *G. J. Fasbina* From the Departments of Pathology and Pediatrics, Southwestern Medical College of the Southwestern Medical Foundation, Dallas, Texas. *Am. J. Dis. Child.* 76, 196-202, 1948

Twenty-one normal infants of approximately similar weight were studied during the neonatal period by means of daily red blood cell counts and hemoglobin determinations, red cell volumes and plasma bilirubin levels. In most of the infants, determinations were made of the bilirubin content in the meconium excreted during the first three days. The velocity constant of excretion of bilirubin was determined in 18 infants by the method of Weech et al. The relation of maternal and fetal isobemagglutinins to the development of neonatal hyperbilirubinemia was investigated in 50 other normal infants.

Evidence is presented which strongly suggests that physiologic hyperbilirubinemia is not purely if at all hemolytic in origin but due mainly to functional immaturity of the liver before and for a variable period after birth. The degree of hyperbilirubinemia could not be correlated with the magnitude of the fall in red cell count and packed red cell volume nor with mother-child ABO and Rh incompatibility. There was an inverse correlation between the amount of bile pigment in the first meconium and the height of the plasma bilirubin rise during the first week of life. Impairment of the bilirubin excretory capacity of the liver was demonstrated in infants with hyperbilirubinemia, whereas normal excretory function was found in infants whose plasma bilirubin levels had returned to normal.

H. W. B.

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## *Contributors to this Issue*

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VIRGILIO CHINI, M D

Professor of Clinical Medicine University of Bari  
Bari Italy

MICHAEL A RUBINSTEIN, M.D

Attending Physician Hematology Montefiore  
Hospital, Senior Clinical Assistant, Hematology,  
Mount Sinai Hospital Clinical Lecturer in Medi-  
cine, Columbia University New York City

J G HURST, A B

Research Fellow Serological Laboratory Office  
of the Chief Medical Examiner of New York City

CLAUDIO MALAGUZZI VALERI, M.D

Assistant Professor of Clinical Medicine, Univer-  
sity of Bari, Bari Italy

ALICE F McBRIDE, B A

Senior Laboratory Technician University of Cali-  
fornia Medical School San Francisco Calif

JOHN C WEAVER, M D

Donner Laboratory University of California,  
Berkeley California

STACY R METTIER, M D

Professor of Medicine University of California  
Medical School San Francisco Calif

IRVING B WEXLER, M D

Clinical Assistant in Pediatrics Jewish Hospital  
of Brooklyn, Associate Transfusionist Adelp-  
hi Hospital Brooklyn N Y

JOHN B MIALE, M D

Laboratories of the Marshfield Clinic and St  
Joseph's Hospital Marshfield, Wis

ALEXANDER S WIENER, M D

Senior Bacteriologist (Serology) to the Office of  
the Chief Medical Examiner of New York City  
Chief Transfusionist, Jewish and Adelp-  
hi Hospitals Brooklyn N Y

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# BLOOD

## *The Journal of Hematology*

VOL IV, NO 9

SEPTEMBER, 1949

### MEDITERRANEAN HEMOPATHIC SYNDROMES

By V CHINI, M.D., AND C MALAGUZZI VALERI, M.D

FROM investigations made during the past ten years in our laboratory and on the basis of several recently published reports, we are now in a position to attempt a classification of Cooley's anemia and allied conditions. We have used the term Mediterranean hemopathic syndromes for a group of blood conditions which have a high incidence among the populations of some Mediterranean countries, and which have in common certain hematologic abnormalities, which represent different varieties of one great group of constitutional and hereditary blood diseases.

To this group belong, among others, two clinically well-defined and easily diagnosed forms, that is, Cooley's anemia, or Mediterranean anemia, and a hemolytic syndrome which about twenty years ago was popularized by Italian authors as hemolytic jaundice with decreased red cell fragility.

Cooley's anemia being universally known, it is superfluous to mention here its clinical features and blood picture. It was first described as a clinical entity by Cooley and collaborators,<sup>67 68 69 72 73 76</sup> and several American<sup>19 271 272 275</sup> and European<sup>62 63 169 199 251 262</sup> authors have widely contributed to its study. On the continent it has been the object of investigations particularly by Caminopetros<sup>22 34 35</sup> and various Italian workers (Cassano<sup>42</sup>, Ravenna and Cannella<sup>211</sup>, Ortolan<sup>182 183 184</sup>, Ortolan and Castagnari<sup>186</sup>, as well as by many others<sup>27 38 46 27 66 77 105 112 129 147 188 191 192 205 206 207</sup>).

The so-called hemolytic jaundice with decreased red cell fragility was first described in Italy by Rietti<sup>212 213</sup> and by Greppi<sup>133 134 135</sup> (1925-1928). Numerous cases have been published subsequently by Italian authors and more recently by others<sup>21 44 61 79 93 94 106 108 109 115 118 141 142 161 164 165 168 190 197 206 211 260 256 266 268 261a 261b 122 167 217 229 270</sup>. It is a familial hemolytic jaundice with a constitutional and hereditary element. Its fundamental feature, and the one which differentiates it from Minkowski-Chauffard's hemolytic anemia (acholuric jaundice), is the presence of an increased instance of the red cells to hypotonic solutions. Other less constant features are hypochromic microcytosis and in nearly all cases ovalocytosis and poikilocytosis.<sup>149 165 197</sup>

From the Clinica Medica University of Bari Bari Italy

Clinically, its differential characteristics are a less intense degree of anemia, less intense and less frequent hemolytic crises, and less favorable results from splenectomy<sup>84 175 135 14\* 166 49</sup>

The clinical and hematologic features of the condition just described have led us to put it in the same general category with Cooley's anemia. Several investigators,<sup>106 10 118</sup> especially American authors (Wintrobe et al.<sup>4</sup>, Atkinson<sup>11</sup>, Dameshek,<sup>81</sup> and others), have interpreted such cases as mild or asymptomatic forms of Cooley's anemia<sup>275 277</sup> and the prevalent opinion now is that there exists one disease entity—that is, Cooley's anemia—which may appear under at least two fundamental forms, that is, (a) *severe* form, the classic Cooley's disease, which develops nearly exclusively in children, has a rapidly fatal course,<sup>\*</sup> and is characterized by an intense degree of anemia, splenomegaly with erythropoietic metaplasia of the spleen, and by typical bone changes, and (b) a *mild* form (hemolytic jaundice with decreased red cell fragility of Italian authors), whose course is less rapid, allowing patients to reach adult age. With this latter form should be grouped a good number of cases which have been described as

Cooley's syndrome or Mediterranean anemia of adults<sup>1 5 9 17 5 49 53 5 80 91 95 100 104 150 15\* 178 187 2 6 75\* 253 766</sup>

Observations in a large series of cases, investigations of the familial element and on the modes of transmission of the fundamental characters of the two conditions, analysis of well-defined clinical and hematologic pictures have allowed us to develop a broader aspect of this group of conditions and a more satisfactory classification of the various forms.

These investigations, which began with the work of Caminopetros<sup>23 24 25</sup> and others,<sup>22</sup> have been further developed by several Italian (Angelini<sup>3</sup>, Micheli and collaborators<sup>155 166</sup>, Pontoni,<sup>207</sup> Gatto<sup>173 124 178</sup>, Chini<sup>51 54 57 179</sup>, Silvestroni and Bianco<sup>277-243</sup>) and American workers (Dameshek,<sup>61 3</sup>, Valentine and Neel<sup>176 177 262</sup>, Smith<sup>74 248</sup>, McIntosh and Wood<sup>16</sup>, Cooley<sup>70</sup>)

By proposing the term Mediterranean hemopathic syndromes we do not intend to suggest that such conditions affect exclusively Mediterranean ethnic groups. Cases have been published recently from other parts of the globe whose clinical and hematologic pictures may be included in this group of diseases.<sup>21 41 82 86 96 97 110 120 132 136 140 219 223 254 760</sup> However, they represent isolated cases, the diagnosis of some of which might be worth reconsidering.

On the other hand, there seems to be little doubt that the conditions under discussion have a particularly high incidence among some populations in the Mediterranean area (Greece, Mediterranean islands, southern Italy, Italian district of Ferrara) and among some ethnically related populations.†

(x) Among the members of families with cases of Cooley's anemia the presence

\* Therapeutic attempts have been made with splenectomy<sup>47a, 52 125 130 139 148 205 248 251</sup>

† Further statistical study may reveal the existence of blood diseases belonging to this group in other countries. In any case it is justified to state that their incidence is by far higher among the inhabitants of some Mediterranean regions. Therefore the suggested term Mediterranean hemopathic syndromes seems to us appropriate and has been adopted for the sake of simplicity.

can invariably be found of subjects (parents, brothers and other siblings) who exhibit some hematologic changes, the most frequent and characteristic being an *increased red cell resistance to hypotonic solutions*. This feature, which was first pointed out by Caminopetros<sup>22 24 25</sup> and by Angelini,<sup>8</sup> has been confirmed by numerous other observations and is now a generally accepted characteristic.

Together with the decreased red cell fragility, other blood changes are found, the most common being hypochromia, microcytosis and leptocytosis. Hypochromic microcytosis with decreased red cell fragility is the fundamental hematologic picture found in siblings of cases of Cooley's anemia (Caminopetros, Angelini, Micheli and collaborators, Gatto, Chini, Silvestroni and Bianco, Smith, Valentine and Neel, Dameshek, and others).

(2) This blood picture, which is also found in siblings of cases of hemolytic jaundice with decreased red cell fragility (Rietti, Greppi, and others), has been termed by Chini as *Mediterranean hematologic disorder*.<sup>55</sup> Valentine and Neel called it mild form of Cooley's anemia or thalassemia minor (thalassemia minima, according to Gatto<sup>127 128</sup>). Silvestroni and Bianco, who found these changes in a number of cases from numerous observations among Italians from the South,<sup>22 227 229 230 231</sup> called it microcytemia.<sup>233 236 237 241 247</sup> However, as it is pointed out by Dameshek and by Smith, a microcytic anemia is in these cases often accompanied by the presence of large, pale, thin macrocytes, whose hemoglobin content is unevenly distributed within the cell (target cells or leptocytes) and which are to be considered as characteristic elements.<sup>248</sup>

(3) Therefore, the Mediterranean hematologic disorder with the same fundamental blood change (decreased red cell fragility) is present in siblings of cases of Cooley's anemia and hemolytic jaundice with decreased red cell fragility.

From the point of view of the nosologic affinity of the two main forms<sup>51 54 228 236 238 239</sup> one can hardly overlook the importance of this common element, and we think it justified to assume that both forms affect subjects who are carriers of the mentioned hematologic taint. This point has been stressed by Gatto,<sup>123 124 128</sup> Chini<sup>51 54</sup> and, as the result of a great number of observations, by Silvestroni and Bianco.<sup>228 236 238 239 241 242</sup>

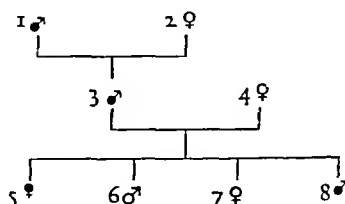
(4) *Carriers of the Mediterranean hematologic disorder are found in those regions and among the populations where Cooley's anemia is incident* (it is probable that many cases go undiagnosed). The disorder represents a necessary early stage for the appearance of Cooley's anemia.

Subjects who are carriers of the disorder may appear to be *quite healthy*, and have no complaints. The disorder is usually detected if it is looked for among families with cases of Cooley's anemia and of hemolytic jaundice with decreased red cell fragility or accidentally as the result of routine investigations. It is apparently transmitted as a dominant characteristic (Gatto<sup>123 124</sup>, Dameshek<sup>82</sup>, Smith<sup>47 248</sup>, Silvestroni and Bianco<sup>40</sup>), and its presence has been followed up in three to four generations of the same family tree.

(5) In many cases the disorder is accompanied by an anemic state. The anemia of these cases cannot be ascribed to any appreciable cause, it is benefited but slightly

by the usual antianemic remedies (liver extracts, iron, blood transfusions), it may last for years or decades, slight splenomegaly may be present. These cases may exhibit very different pictures.

The prevalence in some cases of one or the other morphologic changes may in time permit the nosologic isolation of well-defined syndromes and the use of an appropriate terminology (for instance, the target cell syndrome of Dameshek<sup>11</sup>, the target-oval cell syndrome of the same author, ovalo-poikilocytic hypo-



Fam Br Angelo

FIG 1 —EXAMPLE OF TRANSMISSION OF THE MEDITERRANEAN HEMATOLOGIC DISORDER THROUGH THREE GENERATIONS

TABLE 1 —Example of Transmission of the Mediterranean Hemologic Disorder through Three Generations  
Fam Br Angelo

		Hb	Eryth mill	Color index	Anis	Poik	Ellipt	Target	Simmel test red blood cells resistant to hypotonic saline	
									Mix %	Mo %
Br S 61	1	80	4	0.91	++	++	++	+	66.7	22.3
G I 55	2	72	3.45	1.02					17.4	13
Br A. 35*	3	76	4	0.95	++	++	+	+	64	8
Br M. 33*	4	85	4.2	1.01					12.1	22.1
Br E. 12	5	75	4.5	0.83	++	++	++		36.2	24.4
Br N 9	6	71	4.6	0.77					12.5	20.3
Br F 7	7	71	3.8	0.93					6.1	10.5
Br F 3	8	78	4.6	0.82	++	++	+		36	10.9

\* Cousins

chromic anemia of Micheli and collaborators,<sup>165, 166</sup> and Pontoni<sup>202</sup>, familial microcytic anemia of Strauss and collaborators<sup>254</sup>, constitutional microcytic anemia of Silvestroni and Bianco<sup>227, 231, 236</sup>, etc.) It may well be that some forms, similar to those just mentioned, belong to this group (cases of Fanconi<sup>93</sup>, Cooley<sup>71</sup>, Rundles and Fall<sup>219</sup>, Stransky and Regala<sup>254</sup>, etc.), but it is quite possible that some forms which have been included in it will some day be differentiated and separated. For the time being these forms may be put together as varieties which need to be better known and more satisfactorily classified. Anemia is usually

TABLE 2. — Mediterranean Hemopathic Syndromes. Forms with no Anemia and with Red Cell Changes of Different Type (Carriers of the "Mediterranean Hematologic Disorder")

	Facies	Jaundice	Splenomegaly	Hepatomegaly	Van den Berg's test	Serum Bilirubin mg %	Takata Test	Reticulocytes %	Hemolytic Index (norm = 1)	Serum Fe %	Erythrocytes in millions	Hemoglobin (%)	Color index	Mean corpuscular diameter microns	Mean corpuscular volume cubic micron	Anisocytosis	Poikilocytosis	Elliptocytosis	Target cells	Fragility test		Simmel test red blood cells resistant to hypotonic saline
																				Hemolysis complete	Hemolysis begins	
Misc M 46					D R	Indos 0.50		0.8			5.48	90	0.83			++	++	++	+	0.21	0.46	Simmel test red blood cells resistant to hypotonic saline
Cos R					D R	0.50					4.80	76	0.79	7.48		++	++	++	+	0.28	0.44	54.4
Car C 49		+			D R	1.10				66	5.99	90	0.75							0.29	0.58	54.4
RaC M 43			++	+	D R	0.96	neg			133	5.03	76	0.75	8.28	62	++	++	++	++	0.24	0.46	7.8
Spin T 37	++		++		D R	1.25				100	4.18	80	0.86			++	++	++	++	0.22	0.41	36.5
Sc C 19	++	+	++							166	6.8	72	0.56	6.91		++	++	++	++	0.28	0.42	61.1
Pal C 51	++		++	+	D R	0.78	+				5.60	75	0.66			++	++	++	++	0.22	0.41	—
Fp A 37	++		+		D R	Indos 0.43	neg	0.3			5	78	0.78		66	++	++	++	++	0.22	0.41	20
Cor I 26	++							0.2			4.6	68	0.72		64	++	++	++	++	0.20	0.40	65
Dr F 12	++										4.5	75	0.83			++	++	++	++			19.5
Dr F 3	++										4.6	76	0.82			++	++	++	++			38
Scott A 52	++										5.25	82	0.80			++	++	++	++			14.4
Dr A 34	++										4	76	0.95		67	++	++	++	++	0.26	0.44	35
DIC M 56	++		++	+	Ind	0.38	neg		0.15		4	76	0.95			++	++	++	++			64
Nov F 21	+	+	++		D R	0.88	+	1.2		50	4.9	91	0.95	7.51		++	++	++	++			64
Pass D 57	+	+				Indos					4.4	85	0.95			++	++	++	++	0.26	0.44	25
Dr S 61	+										4.2	80	0.91	7.95		++	++	++	++	0.26	0.44	33.7
DIMas F	+		+	+	D R	0.74	neg			166	4	74	0.92			++	++	++	++	0.26	0.48	70

TABLE 3.—*Mediterranean Hemopathic Syndromes Forms with Anemia of Different Type*

	Facies	Jaundice	Splenomegaly	Hepatosplenomegaly	Van den Bergh test	Serum Bilirubin mg %	Taka's Test	Reticulocytes %	Hemolytic Index (norm = 1)	Serum Fe %	Erythrocytes in millions	Hemoglobin (%)	Color Index	Mean corpuscular diameter microns	Mean corpuscular volume cubic micron	Anisocytosis	Poikilocytosis	Elliptocytosis	Target cells	Fragility test		Simmel test red blood cells resistant to hypotonic saline		
																				Hemolysis complete	Hemolysis begins			
B L 37	+				ind	0.44				85	3.86	47	0.60							+	0.28	0.48	34.3	17.1
Copp C					D R ±	0.39					3.5	71	1.02	7.24						+	0.30	0.48	22.4	22.8
Ferr G 39	+		+	+	D R	0.96					3.7	60	0.81			+	+						59.3	24.4
Nor P 27	+		+	+	ind	0.40		0.8	1.8		3.83	68	0.89		96	+	+						32.1	8.5
Ferr A	+	+			ind	0.40				133	2.94	26	0.44		67	+	+				0.26	0.50	35.5	6.8
Pier F 62		+	+		ind	0.56				100	3.2	42	0.65	6.85		+	+				0.26	0.52		
Los M 27					ind	1.26		0.05			3.45	70	0.99			+	+				0.26	0.44	76.4	11.7
Ris P 58					ind						3.55	75	1.05			+	+							
Copp A					ind	0.37					3.4	72	1.06	7.34	80	+	+				0.26	0.50	38.2	14.7
Palm B 50											3.2	68	1.05			+	+						59.3	15.6
Test F										83	3.89	65	0.85			+	+				0.26	0.50	52.5	12.6
Sail L 42																								

markedly hypochromic, less frequently hyperchromic (Patrassi and Taglioni<sup>197</sup>, Chini<sup>55</sup>, Muratore<sup>174</sup>) In the hypochromic cases there is nearly always microcytosis, with hyperchromia, macrocytosis may also be found In nearly all cases marked ovalocytosis is observed, in many cases aniso-poikilocytosis is prevalent, and in some we find schistocytosis similar to that found in Cooley's anemia<sup>74 155 166 182</sup>, that is to say, morphologically one finds a blood picture very much resembling Cooley's anemia, with the exception of circulating erythroblastosis

We can, therefore, refer to these various pictures as *varieties of the anemic form of the Mediterranean hematologic disorder* Some of these varieties are poorly defined, others resemble very nearly Cooley's anemia

The clinically well-defined varieties of this anemic form are usually hypochromic, microcytic, ovalocytic, aniso-poikilocytic and schistocytic Besides, in many cases,<sup>173 247 248 261a 261b 274</sup> stippled cells and target cells (Dameshek) are present but the latter do not appear to be characteristic of these forms<sup>18 24 87 88 137 202 220</sup> There is marked hyperplasia and erythroblastic anaplasia of the bone marrow<sup>27 84 122 143 183</sup>

In spite of discordant views,<sup>118 221</sup> a distinction has to be made here between this type of anemia—even its hypochromic microcytic variety—and achylic hypochromic anemia (idiopathic hypochromic anemia), differentiating points being its ethnographic distribution, the high iron content of the blood (Chini, and Perosa<sup>60</sup>, Perosa<sup>201</sup>), the nearly absent response to iron therapy, the usual presence of normal gastric secretion,<sup>64</sup> the absence of some clinical signs which are usually found in idiopathic hypochromic anemia, such as glossitis, dystrophic changes of the fingernails, Plummer-Vinson syndrome<sup>64</sup>

(6) In numerous cases of Mediterranean hemopathic syndrome we find a *marked hemolytic element* These cases are more readily recognizable clinically and have been termed hemolytic jaundice with decreased red cell fragility (Rietti<sup>212 213 215 216</sup>, Greppi<sup>133 134 136</sup>) The picture is that of the Mediterranean hematologic disorder with anemia of one or the other variety (increased resistance to hypotonic solutions, occasional ovalocytosis,<sup>214</sup> presence of target cells oval-target cell syndrome of Dameshek<sup>82</sup>) There is marked hyperplasia and erythroblastic anaplasia of the bone marrow, but no erythroblasts are found in the circulating blood and hemolysis is increased In some cases the hemolytic index reaches values as high as in acholuric jaundice<sup>55 172</sup>

Jaundice is present, there is marked splenomegaly and intense hemolytic crises may occur The hemolytic disturbance may respond effectively to splenectomy<sup>62 172</sup> Histologically, the spleen shows changes of the hemolytic type with no erythropoietic metaplasia, which is a differentiating point from Cooley's anemia The main feature in these cases is the increased hemolysis, a point which should not be overlooked in diagnosis and treatment (splenectomy)<sup>2 3 135 142 166</sup> Operation is followed by a reduced hemolysis, but there is no effect on the morphologic changes, which in some cases are even more accentuated after splenectomy<sup>5\* 53</sup> In fact, some cases after splenectomy show a marked and persistent erythroblastemia<sup>2 3 75 74</sup> The postoperative picture resembles more closely that of Cooley's



TABLE 4—Mediterranean Hemopathic Syndromes. Forms with Hemolytic Jaundice (Hemolysis, Jaundice with Decreased Red Cell Fragility of Italian Authors)

	Facies	Jaundice	Splenomegaly	Hepatomegaly	Van den Bergh test	Serum Bilirubin mg. %	Takata test	Reticulocytes %	Hemolytic Index (norm = 1)	Serum Fe %	Erythrocytes in millions	Hemoglobin (%)	Color index	Mean corpuscular diameter microns	Mean corpuscular volume $\mu$	Anisocytosis	Poikilocytosis	Elliptocytosis	Target cells	Fragility test		Stimel test red blood cells resistant to hypotonic saline
																				Hemolysis complete	Hemolysis begins	
Pant N 20	++	++	++	++	D R $\pm$ Ind	5.77	+	1.7	35	166	3.81	41	0.53	8.11	44	+++	++	++	++	0.21	0.48	3+ 0 9.5
DeCes L 23	++	++	++	++	D R $\pm$ Ind	3.33	+	0.8	23 0	83	3.2	55	0.87		73	++	++	++	++	< 0.18	0.70	50 1 31
Gal T 59	+	++	+	+	Ind	1.95	++		15.8	166	2.9	55	0.95	8.58	84	++	++	++	++	0.22	0.46	45 5 17.2
Strip M 34		++	+	+	Ind	2.66	++	0.7	14 9	166	5.25	74	0.70	8.30	64	++	++	++	++	0.28	0.54	48 9 21.6
Arm M 13	++	++	++	+	Ind	2.14	++	4.2	9.2	166	3.3	54	0.82	7.73	91	++	++	++	++	< 0.22	0.48	71.8 5.8
For L 23		++	++	++	Ind	1.62	++		7.7		2.62	40	0.77		72	++	++	++	++	0.28	0.44	43.3 16.6
For S		++	++	++	Ind	1.77	++		3.82		1.93	46	1.20		114	++	++	++	++	0.28	0.44	64.2 17.6
Verd R 26	++	++	+	+	D R $\pm$ Ind	1.08	+		3.1		3.5	51	0.73			+	++	+	+	0.26	0.42	54 17.2
Bal R 44	+	++	+	+	D R $\pm$ Ind	3.10	+	+	9.6	100	4.72	72	0.75		75	+++	++	++	++	0.26	0.46	71.4 4.5

\* Splenectomy

TABLE 5 — *Mediterranean Hemophagic Syndromes: Forms with Slight Jaundice and with More or Less Normal or Presumably Normal Hemolysis*

Facies	Jaundice	Splenomegaly	Hepatomegaly	Van den Berg's Test	Serum Bilirubin mg %	Takata Test	Reticulocytes	Hemolytic Index (norm = 1)	Serum Fe %	Erythrocytes in millions	Hemoglobin (%)	Color index	Mean corpuscular diameter microns	Mean corpuscular volume cubic micron	Anisocytosis	Poikilocytosis	Elliptocytosis	Target cells	Fragility test		Simmel test re-blood cells resistant to hypotonic saline
																			Hemolysis complete	Hemolysis begins	
Tom F 21	+	+	+	D R ±	3.50	+	0.3	2.2	100	6.2	85	0.69	7.29	63	++	++	++	++	0.24	0.41	43
DIG I 20	+	+	+	D R ±	3.02	+	0.9	2.2	100	4.94	62	0.61	7.29	63	++	++	++	++	0.20	0.42	40
DeC S 19	+	+	+	D R ±	2.29	neg	1.2	2.07	83	3	65	0.83			++	++	++	++	>0.18	0.50	25
LaC C 18	+	+	+	ind	2.29	+				1.55		0.60			++	++	++	++	0.22	0.45	
Dip C	+	+	+	D R ±	1.99	+			100	4.5	.2	0.78	6.67	62	++	++	++	++	0.21	0.46	47
Venez G 21	+	+	+	D R ±	1.41	+			133	5.12	77	0.75			++	++	++	++	0.26	0.48	51
Max C	+	+	+	ind	1.35					2.10	35	0.85	8.11		++	++	++	++	0.22	0.40	12
Sard	+	+	+	D R ±	1.33				133	5.40	78	0.72			++	++	++	++	0.22	0.40	82
Bald M 18	+	+	+	ind	1.11		0.5			4.25	41	0.47			++	++	++	++	0.26	0.52	54
Val A 26	+	+	+	D R ±	1.32	neg	5	0.5		3.78	42	0.55		62	++	++	++	++	0.22	0.50	29

\* Metrorrhagia

anemia chiefly on account of the erythroblastemia and in some cases because of bone lesions as seen in the x-ray films <sup>2 3 53 202</sup>

(7) Besides these varieties of the Mediterranean hematologic disorder with jaundice and hyperhemolysis, cases are seen in which jaundice is evident but no increased hemolysis is found. The hemolytic index of these patients is normal or even lower than in normal cases, in spite of the obvious presence of jaundice and of the high values of bilirubin in the blood by the indirect test. In the absence of increased hemolysis we cannot term them as cases of hemolytic jaundice. These cases have some points in common with the so-called *juvenile intermittent jaundice of Meulengracht or nonhemolytic prehepatic jaundice*<sup>84 92 181 194 195 196</sup> and they represent a fairly large number of cases of Mediterranean hemopathic syndromes (Patrassi and Taglioni<sup>197</sup>, Cassano and Benedetti<sup>45</sup>, Chini<sup>54</sup>, Malaguzzi-Valeri<sup>149</sup>, Castaldi and Leonardi<sup>121</sup>)

It has been suggested by various authors<sup>45 54 197 208</sup> and recently confirmed by the work of Perosa on hemoglobin tolerance curves, that in subjects affected with this type of jaundice, there is a derangement of the liver function in the sense that the transformation of bilirubin from the indirect to the direct form and its subsequent elimination from the blood do not take place.

A hepatic factor of this type may be present even in the full fledged case of hemolytic jaundice with decreased red cell fragility, in which a more or less marked hyperhemolysis is found. In fact, in these cases one often finds a discrepancy between the hemolytic index and the values of the indirect bilirubin of the blood, and also a delayed direct Van den Bergh reaction<sup>84 79 116 197 201</sup>. These findings have been interpreted in various ways, for instance, they have been ascribed to liver dysfunction, to the presence of an abnormal pigment not detected by Nencki's reagent, etc. (investigations on this subject are being carried out in our Institute)

(8) All these different forms have some common fundamental elements which permit us to consider them as belonging to one great group. These elements are (a) Their nearly exclusive limitation to Mediterranean people, (b) The presence of a constitutional, familial and hereditary element, (c) The increased resistance of the red cells to hypotonic solutions.

Therefore, we find it justified to collect them in one group under the term of Mediterranean hemopathic syndromes, and to consider the Mediterranean hematologic disorder as their fundamental pathogenetic factor.

The pathogenesis of the single clinical pictures is still very obscure.

On the basis of our present knowledge, mainly from the analysis of the familial tendency of these syndromes, it seems to us that something can be said on the pathogenetic connections between the so-called Mediterranean hematologic disorder and Cooley's anemia and on the etiopathogenesis of the so-called hemolytic jaundice with decreased red cell fragility.

With regard to Cooley's anemia, one important point has been brought to evidence from the study of the parents of the patients, and that is that where the investigation has been adequate, *both parents* of an individual affected with Cooley's

TABLE 6—*Mediterranean Hemophagic Syndromes* Forms with jaundice but with no increased Hemolysis

	Facies	Jaundice	Splenomegaly	Hepatomegaly	Van den Bergh Test	Serum Bilirubin mg %	Takata Test	Reticulocytes	Hemolytic Index (norm = 1)	Serum Fe %	Erythrocytes in millions	Hemoglobin (%)	Color Index	Mean corpuscular diameter microns	Mean corpuscular volume cubic micron	Anisocytosis	Poikilocytosis	Elliptocytosis	Target cells	Fragility test		Simmel test red blood cells resist ant to hy potonic saline		
																				Hemolysis complete	Hemolysis beginning	Max %	Min %	
Pap C 22	+	++	++	+	D ind	3.77	neg	2	0.51	50	5.2	82	0.78		69	++	++	++	+	0.26	0.46	55.8	19	VII/39
Ferr P 23	+	++	++	++	ind	2.20			0.87	366	3.54	50	0.70		72	++	++	++	+	0.22	0.46	45.7	19.9	VIII/39
					ind	2.27			1.22							++	++	++		0.22	0.42	51.7	14.9	VIII/39
					ind	2.96			1.0*			55				++	++	++		0.20	0.46	66.8	21.1	XI/39
					D R ind	3.25	neg	1.4	1.24		3.20	55	0.85	7.30	65	++	++	++		0.20	0.46	56.2	15.6	IV/41
					ind											++	++	++		0.20	0.42	56.6	9.9	VI/41
Ventr VI 42	++	++	+	+	ind	2.36	+	0.1	2.58	183	5.38	70	0.66	7.54	52	+	++	++	++	0.26	0.46	36.1	9.8	XI/46
					ind	2.22			1.36		5.47	80	0.74	7.68	74					0.26	0.48	41.1	15.6	IV/47

\* Splene-tomy

anemia have been found to be carriers of the Mediterranean hematologic disorder

It was Caminopetros<sup>34 35</sup> who first called attention to this point, even if he did not stress it as a fundamental feature in the familial tendency of the disease, and it was confirmed by Angelini<sup>8</sup> and later by Micheli, Penati, Momigliano-Levi<sup>115</sup> and others (Panoff<sup>189</sup>)

In 1939, Chini,<sup>51 52</sup> on the basis of the reports from Caminopetros, Angelini and Micheli, stressed the unusual fact of the presence of a hematologic taint in both parents of patients suffering from Cooley's anemia and stated that the findings could not be ascribed to mere coincidence.<sup>51 57</sup>

Subsequently (1941), further reports from other authors (Ortolani and Vallisneri<sup>187 262</sup>, Wintrobe and collaborators<sup>274</sup>, Atkinson<sup>14</sup>, Pehu and Leriche<sup>199</sup>) led Chini<sup>51</sup> to state that this bilateral hereditary tendency was to be considered as a fundamental factor in the pathogenesis of Cooley's anemia

Independently, in 1941-1942, Gatto<sup>123 124</sup> made his first report on the results of his investigations on the members of the families of 8 cases of Cooley's anemia. His conclusions were that increased resistance of the red cells to hypotonic solutions and microcytosis, as a rule hypochromic and accompanied by ovalocytosis and poikilocytosis, were constantly present in both parents of patients with Cooley's anemia. According to Gatto, this trait (hyperresistant microcytosis)

is a dominant hereditary characteristic which is carried as a heterozygous gene and Cooley's anemia develops only in subjects whose parents are both affected with the disorder and who carry the hematologic characteristic as a homozygous gene. The bilateral hereditary element in these cases has been subsequently confirmed by various authors (Pierce<sup>204</sup>, Valentine and Neel<sup>178 177 262</sup>, Dameshek<sup>82</sup>, Smith<sup>247 248</sup>, McIntosh and Wood<sup>162</sup>, Trincao<sup>260</sup>, etc.<sup>18</sup> and recently in Italy by Burgio<sup>97</sup>, Careddu and Magrassi<sup>41</sup>, Silvestroni and Bianco) in a large number of cases.<sup>338 341</sup>

In Gatto's opinion, Cooley's anemia is an example of *dominant hereditary characteristic with lethal homozygous effect*

In the present state of our knowledge this seems to be the fundamental fact which has been agreed upon from the study of the familial tendency of Cooley's anemia and allied syndromes

It would therefore appear that Cooley's anemia represents the most severe of the Mediterranean hemopathic syndromes. Its severity seems to be caused by the lethal homozygous effect of the presence of the trait in both parents.\*

\* As Chini pointed out in 1941, if it is true that typical Cooley's anemia only affects those subjects whose parents are both carriers of the Mediterranean hematologic disorder, then we should not use the term of mild forms of Cooley's anemia to indicate the various forms of Mediterranean hemopathic syndromes or the cases of apparently healthy carriers of the Mediterranean hematologic disorder. In cases of Mediterranean hemopathic syndromes only one of the parents is a carrier. In a typical case of Cooley's anemia we never see a gradual attenuation of the symptomatology, so as to make it possible to identify the case with one or the other variety of the Mediterranean hemopathic syndromes, and we never see a case of Mediterranean hemopathic syndrome becoming so severe as to resemble a typical case of Cooley's anemia. One could use the term of mild forms in these cases as suggested by some American authors, if there existed a transition in some of them from the mild to the severe form. This

Of this trait we know only some features which are more readily detectable, the decreased red cell fragility and often the hypochromic microcytosis, also, according to Dameshek,<sup>61</sup> the target cells or leptocytes and, according to Smith,<sup>248</sup> the thin and pale macrocytes and the stippled cells. These physical and morphologic changes reveal the presence of a more profound structural disorder of the red cell whose essence is still obscure (Pontoni<sup>208</sup>, Chini<sup>64</sup>, Rietti<sup>216</sup>). Other characteristics of the trait (first described by Caminopetros<sup>24 36</sup> and in Italy by Gatto,<sup>123 124 126</sup> followed by Chini,<sup>61 64</sup> Silvestroni and Gentili<sup>242</sup>) are in the somatic line. The presence of the Mediterranean hematologic disorder is frequently found in subjects with high and thick zygoma cheek bones (*facies microcytica*, according to Silvestroni and Gentili<sup>242</sup>). What we have often found is an increased distance between the zygomas. However, it should be noted that this characteristic is frequently found among the population of Southern Italy<sup>64 68</sup> and that on the other hand subjects who exhibit a mongoloid face may not be carriers of the Mediterranean hematologic disorder, though they may be affected with some other blood disturbance.

With regard to hemolytic jaundice with decreased red cell fragility, here also some family members of the patients are carriers of the Mediterranean hematologic disorder, though only one parent is affected and not both as in Cooley's anemia.

In some family members of cases affected with this condition, a constitutional hyperhemolytic state is also found, either in the same family side of the carrier or in the opposite.<sup>171</sup>

This characteristic is in some cases quite evident,<sup>44 171</sup> in others only slightly pronounced (slight increase of the red cell fragility, and at the same time an in-

view finds confirmation in the clinical analysis of Cooley's anemia and of those Mediterranean hemopathic syndromes whose characteristics are well-defined. In a sense the terminology suggested by Valentine and Neel<sup>262</sup> of thalassemia major (Cooley's anemia) and thalassemia minor seems to us more appropriate though thalassemia minor would seem to include well-defined syndromes some of which are of marked severity and for which the term minor could hardly seem acceptable. It should be noted that for the carrier state Gatto uses the term thalassemia minima.<sup>127 128</sup> While the view expressed in this paper has on one hand more consideration of the genetic factors whose etiopathogenic nature has been established on the other hand it does not disregard the importance of the clinical characteristics: our scheme of classification is very similar to that of Dameshek.<sup>42</sup> Cooley's anemia represents the extremely severe and the fatal among the Mediterranean hemopathic syndromes. It is a constitutional familial and hereditary condition but it is not transmissible because its appearance (lethal homozygous effect) makes procreation impossible. At least this is what we know from nearly all cases that have been described (infantile prepubertal mortality). With regard to this point a re-examination of all the published cases of Cooley's anemia in adults would seem necessary in order to make a detailed assessment of their genetic elements and to find out whether there did or did not exist bilateral hereditary factors. For the cases of Cooley's anemia in adults which have been published (two personal cases included) Chini has suggested the term of syndromes of the Cooley type.<sup>49 52</sup> It is a subject still open to investigation. The term mild forms of Cooley's anemia could be used for the brothers of a typical case of Cooley's anemia whose parents are both carriers of the Mediterranean hematologic disorder and who exhibit clinical hematologic x-ray and histologic pictures (erythrocytic metaplasia outside the bone marrow) which very nearly resemble even in their degree of severity typical Cooley's anemia. Such cases as these have been published.<sup>14 15 25 150</sup>

crease of the maximal resistance, spherocytosis) Some authors have suggested the term of mixed forms of hemolytic jaundice, and in a wide sense, of hemolytic diathesis, common to both forms<sup>44 266 267</sup> However, the observations with regard to this group of cases are still isolated and it is not possible at present to come to any conclusions

It is quite possible that the presence of a constitutional hemolytic factor may be of importance in the pathogenesis of some Mediterranean hemopathic syndromes which show a marked hemolytic element This should be more common in those regions where the Mediterranean hematologic disorder and hyperhemolysis are comparatively frequent events And such is the case in some districts of Southern Italy, the origin of the majority of the presently published cases of "hemolytic jaundice with decreased red cell fragility

This, however, is not the only factor which may be responsible for the hemolytic element of the condition, the structural changes in the red cells may play their role (microschistocytosis<sup>155 156 157</sup>) or the cause may lie in a combination of various morbid factors, as well as in splenopathic conditions, in a wide sense In this respect, we can hardly overlook the importance of malaria, whose role has also been discussed with regard to Cooley's anemia<sup>70 26 39 50 51 58 90 128 151 160 191 225</sup>

A hemolyzing action of blood plasma has been found by Frontali and Rasi<sup>117</sup>, however, this has not been confirmed by Chini and collaborators, who could detect it only in cases in which the blood cholesterol values were very low<sup>20 58 102</sup>

The question is still very obscure We still do not know why and how carriers of the hematologic characteristics of acholuric jaundice at a certain time become affected with hyperhemolysis In some cases we find an intercurrent illness, in others the real cause cannot be found, the hemolytic character of the condition being then termed idiopathic

Very little has been known regarding factors which determine or help in the transition from the simple stage of hematologic disorder to that of anemia in its different varieties (ovalo-poikilocytosis, etc.) Occasional factors, such as infectious diseases, hemorrhages, abundant menstruations, food deficiencies, endocrine disturbances, etc., may contribute, but constitutional and hereditary elements of various type may intervene, and this side of the question is now being widely investigated (significance of ovalocytosis, presence in some family groups of some other hemopathic condition, possible influence of malaria on carriers of the trait)<sup>54 55 222 234</sup>

With regard to the fundamental structural derangement of the red cells which is the cause of their abnormal physical sedimentation rate,<sup>154 158</sup> etc., and morphologic state, the question is still obscure

As has been said before, there probably exists a profound biochemical alteration of the red cells which is most intense in Cooley's anemia, but may be present in a lesser degree in the other Mediterranean hemopathic syndromes

As suggested by Whipple and Bradford<sup>271 272</sup> for Cooley's anemia, in the other syndromes of the group there is also possible the presence of an abnormal capacity

for utilizing iron and elaborating hemoglobin. This hypothesis is supported by the presence of erythroblasts with a red fluorescence in the bone marrow (Freudenberg and Esser<sup>113, 114</sup>) and by other abnormalities in the metabolism of porphyrin (Lichtwitz<sup>148</sup>, Vannotti<sup>254</sup>, Tropp and Penef<sup>261</sup>), by the high values of blood iron in spite of the constant and in some cases quite marked hypochromia (Perosa<sup>201</sup>, Chini and Perosa<sup>60</sup>, Amato<sup>6</sup>, Cartwright<sup>42</sup>), and by the variations in the resistance of hemoglobin to alkaline denaturation in Cooley's anemia (Vecchio<sup>255</sup>, Bianco<sup>22</sup>, Putignano and Fiore-Donati<sup>209</sup>). It appears, however, that there is no change in the crystallographic and spectrophotometric characteristics of hemoglobin (Marmont and Bianchi<sup>159, 160</sup>).

In spite of the methodical work of Bussi<sup>29</sup> and of Astdal and collaborators,<sup>10, 11, 12, 13</sup> on erythroblastometric curves and on bone marrow maturation, there is no conclusive evidence of the significance of microcytosis and on the question of its interpretation as a congenital abnormality.

Lehndorff,<sup>145</sup> Caminopetros<sup>25</sup> and others (Chini<sup>51</sup>, Gatto<sup>123, 124, 128</sup>, Fanconi<sup>89</sup>, Heilmeyer<sup>128</sup>, etc.) favor the hypothesis that the hematologic abnormality which is fundamental in Cooley's anemia, that is the Mediterranean hematological disorder, is due to a process of mutation in some groups of Mediterranean populations.

That a process of mutation may have particularly affected some Mediterranean groups which are still recognizable from their facial configuration (width and thickness of the zygoma, cheek bones) was pointed out by Gatto in 1942 and recently confirmed by the results of investigations carried out by Graziosi,<sup>131</sup> on fossilized skulls from the superior paleolithic ages found in Sicily.

The high incidence of the Mediterranean hematologic disorder in some regions (investigations carried out among Americans of Italian descent by Valentine and Neel<sup>176, 177</sup>, in Italy by Silvestroni and Bianco<sup>229, 230, 241</sup>, Careddu,<sup>40</sup> Careddu and Magrassi<sup>41</sup>, Leonardi and collaborators<sup>145</sup>, Bianco<sup>22</sup>, our own investigations still under way, by Banton<sup>15</sup> in Cyprus), and the etiopathogenetic connections of the disturbance with the various Mediterranean hemopathic syndromes, including Cooley's anemia, represent a subject of great social importance. The widespread diffusion and the intensity of the morbid characteristics which are transmitted to their descendants by the carriers of the trait had been stressed by Chini<sup>57</sup> in 1939 and particularly emphasized by Caminopetros (1937, 1938). Such diffusion is now being more widely revealed through large scale investigations on the incidence of the Mediterranean hematologic disorder among the population of some districts of Italy.

Caminopetros<sup>25</sup> had suggested the necessity of advising the carriers of the trait against marriage. The recently established evidence that Cooley's anemia only appears in individuals whose parents are both carriers of the Mediterranean hematologic disorder is of great importance in this respect, and especially if it receives further confirmation, will allow for a less extreme view with regard to marriage limitations. In fact, as suggested by Silvestroni and Bianco,<sup>241</sup> and as we



have been advising for some time to the family members of our own cases, it would be sufficient to discourage marriage between persons who are both carriers of the disorder

Paleontologic investigations have revealed the presence of particular skeletal lesions, especially of the skull, in the skeletons of individuals belonging to races now nearly completely extinct

The analysis of skulls found in ancient cemeteries or belonging to mummies still in a state of very good preservation, of skeletons from ancient native populations of America, Incas from Peru (Williams<sup>79</sup>), Indians from Colombia (Feingold and Case<sup>101</sup>), Aztecs from Mexico, Maya Indians from Yucatan (Moore<sup>170</sup>) (it should be noted that the Indians from the northern parts of South America and those from Mexico appear to have a common origin) in some necropolises from Arkansas (Wakefield and collaborators,<sup>68</sup> etc.), has shown the existence of the same typical and unmistakable skeletal lesions which we now find in individuals suffering from sickle cell anemia, Cooley's anemia, or, to a lesser extent, in cases of Mediterranean hemopathic syndromes (Perosa and Viterbo, etc.) Not without foundation do students of paleontology believe that the extinction of those ancient populations was contributed to by the high incidence among them of some blood diseases which probably developed through processes of human mutation on a widely hereditary and familial basis (Williams and Moore) The recent report of cases of sickle cell anemia among some native populations of Mexico, whose anthropologic characteristics are very similar to those of ancient Aztecs (Wallace and Killingsworth<sup>69</sup>) has led some authors to believe that those remnants of a disappearing race have inherited from their ancestors some genetic characteristic.

We still do not know whether such bone lesions are to be ascribed to blood diseases similar to sickle cell anemia or to Cooley's anemia or to some other hemopathic condition with a more or less accentuated hemolytic character (bone lesions, which radiologically resemble those found in Cooley's anemia are also seen, and in some cases quite marked, in acholuric jaundice and in other forms of hemolytic anemia [Gänsslen<sup>119</sup>, Caffey<sup>12</sup>, Perosa and Viterbo, etc.<sup>61 76 85 218 211</sup>]), or even to nonhemopathic conditions The study of the paleolithic skulls found in the caves of St. Teodoro, Sicily, has revealed the presence in them of diffuse osteoporosis (Gatto, Graziosi) Osteoporosis of this type has been observed by Adachi<sup>4</sup> in prehistoric skulls in Egypt Owing to war restrictions and to lack of proper equipment, we have been unable to carry out a group of investigations which we had planned here in Puglia (necropolis of Canne) We are not aware that other investigations of this kind have been carried out elsewhere, except those of Caponnetto<sup>13</sup> on 25 skulls belonging to the Anatomical Museum of Catania, Sicily In 13 of them the author found osteoporosis, in some cases a moderate degree of radial striation of the skull

It can be assumed with some foundation that the diseases—probably blood conditions—which had been the cause of the characteristic lesion of the skeleton, contributed to the gradual extinction of those ancient races

Paleontology thus throws light on the history of disappeared populations offer

ing new possibilities of interpretation. It can lend justification to the warning of Caminopetros, because there is no doubt that the spreading of the Mediterranean hematologic disorder with its dominant character will inevitably lead, through an increase in the number of marriages between carriers of the disturbance, to the appearance of an always increasing number of cases of Mediterranean hemopathic syndromes and of fatal cases of Cooley's anemia.

The study of the Mediterranean hemopathic syndromes represents a great chapter open to research which involves difficult problems of clinical, historical and social importance.

## REFERENCES

- <sup>1</sup> ACUÑA M. Alteraciones radiológicas del esqueleto en la ictericia hemolitica congenita. *Prensa med argent* 24, II 1878 1937
- <sup>2</sup> — RÉSULTATS Éloignés de la splenectomie dans l'anémie érythroblastique. *ref Arch med Enf* 41 395 1938
- <sup>3</sup> — Results of splenectomy in two cases of erythroblastic anemia. *ref Am J Dis Child* 65 813 1943
- <sup>4</sup> ADACHI B. Die Porosität des Schädeldaches. *Ztschr f Morph u Anthropol* 7 373 1904 (quot by Busacchi)
- <sup>5</sup> ALLEN E G AND CHILDS D S. Erythroblastic anemia. *New York State J Med* 36 641 1936
- <sup>6</sup> AMATO M. Considerazioni sul comportamento del ferro serico in alcuni casi di anemia di Cooley. *Riv Clin Pediatr* 44 711 1946
- <sup>7</sup> ANDRUS W D AND HOLMAN C W. Splenectomy in various blood disorders. *Ann Surg* 109 64 1939
- <sup>8</sup> ANGELENTI V. Primi risultati di ricerche ematologiche nei familiari di ammalati di anemia di Cooley. *Min Med* 28 II 331 1937
- <sup>9</sup> ARAVANTINOS A. AND DELIANNIS G. Ueber das Auftreten der Erythroblastenanämie bei Erwachsenen. *Klin Wchnschr* 15 II 1792 1936
- <sup>10</sup> ASTALDI G, GALLO V AND TOLENTINO P. Ricerche sulla genesi della microeritrocitosi. *Policl infant* 15 193 1947
- <sup>11</sup> — AND — Ricerche sulla genesi della microeritrocitosi. (I) Studio eritroblastometrico della Iishmaniosi infantile. *Policl infant* 16 Suppl 1 44 1948
- <sup>12</sup> — AND REGGIANI C. Ricerche in vitro sulle possibilità maturative dei reticulociti e degli eritroblasti del sangue del morbo di Cooley. *Boll Soc it Biol sper* 22 481 1946
- <sup>13</sup> — AND — Ricerche comparative sulla maturazione degli eritroblasti e dei reticulociti del midollo umano normale e del midollo del morbo di Cooley. *Boll Soc it Biol sper* 22 714 1946
- <sup>14</sup> ATKINSON D W. Erythroblastic anemia. Report of two cases in adult siblings. *Am J M Sc* 198 376 1939
- <sup>15</sup> AURICCHIO L. Su alcune sindromi di anemia con splenomegalia a carattere familiare nell'infanzia. *Pediatr* 36 1023 1928
- <sup>16</sup> BANTON A H. Disc on Rubie Rep. *Proc Roy Soc Med* 41 42, 1948
- <sup>17</sup> BARASCIUTTI A. AND ZEN G. Sulla sindrome di Cooley nell'adulto. *Giorn Clin Med* 28 75 1947
- <sup>18</sup> BARRETT A M. A special form of erythrocyte possessing increased resistance to hypotonic saline. *J Path & Bact* 46 603 1938
- <sup>19</sup> BATT J M, BLAKFAN K D AND DIAMOND L K. Blood studies in infants and children. *Am J Dis Child* 43 667 1932.
- <sup>20</sup> BENEDETTI G AND MERLO P. Ricerche su l'anemia da malaria. *Riv Clin Med* 41 -79 1940
- <sup>21</sup> BETTONI I. Ictero emolitico familiare con aumento delle resistenze osmotiche dei globuli rossi. *Min Med* 26 II 278 1935
- <sup>22</sup> BIANCO I. Sulla resistenza emoglobinica nei portatori di microcitemia e di falcemia. *Policl (Sez Prat)* 55 103 1948
- <sup>23</sup> — Sulla frequenza della microcitemia in alcuni paesi delle Puglie. *Min Med* 39 I 2—, 1948

- <sup>24</sup> BOHRD M G The significance of target cells in anemia Am J M Sc. 202. 869 1941
- <sup>25</sup> BUFANO, M. Sindrome eritremica intervenuta dopo splenectomia in un caso di anemia emolitica splenomegalica Folia med 22. 387, 1936
- <sup>26</sup> — Le influenze dell'endemia malarica sulla emopoiesi e sulle emopatie Giorn Clin Med 21. 180 1939
- <sup>27</sup> BUROIO, G R Il mielogramma nei genitori di bambini ammalati di malattia di Cooley Boll. Soc. it Biol sper 29 393, 1945
- <sup>28</sup> BUSACCHI, V Esisteva il morbo di Cooley nell'America precolombiana? Castalia 2. 465 1946
- <sup>29</sup> BUSI L Sulla genesi della microcitosi vera nel cosiddetto ittero emolitico con resistenze globulari aumentate Sett Med 34. 124 1946
- <sup>30</sup> — AND SCHIAVINI A Contributo emato-chimico alla conoscenza dell'ittero emolitico a resistenze globulari aumentate Min Med 37 I 165 1946
- <sup>31</sup> BYWATERS E G L. The Cooley syndrome in an English child Arch Dis Childhood 13 173 1938
- <sup>32</sup> CAFFEY J The skeletal changes in chronic hemolytic anemias Am J Roentgenol 37 293 1937
- <sup>33</sup> CAMINOPETROS, J Erythroblastiké Anaimia (gr.) Kliniké 24 1935
- <sup>34</sup> — L'anémie erythroblastique des peuples de la Méditerranée orientale Monogr Accad d'Athènes 6 III 1937
- <sup>35</sup> — Recherches sur l'anémie erythroblastique infantile des peuples de la Méditerranée orientale Ann de Méd 43 27 104 1938
- <sup>36</sup> CAPONNETTO A. Studio anatomico-radiologico delle strutture dei parietali nel confronto dei caratteri disomorfici descritti nei familiari degli eritremici Boll Soc med chir Catania 7 441, 1939.
- <sup>37</sup> CAREDDU, G Anemia splenica infantile e terapia atonica Riv Clin Pediatr 27 1 1929
- <sup>38</sup> — Osservazioni sull'anemia di Cooley in gemelli Studi Sassari med 18 3, 1940.
- <sup>39</sup> — L'eritroblastosi periferica nella anemia di Cooley Arch Ital Pediatr 9 218, 1942.
- <sup>40</sup> — Modificazioni costituzionali delle resistenze globulari e della bilirubinemia in zone di speciale frequenza della malattia di Cooley Arch Ital Pediatr 10 280 1943
- <sup>41</sup> —, AND MAORASSI F Studi sulla costituzione ematologica dei sardi Boll Soc. it Biol sper 22 325 1946
- <sup>42</sup> CARTWRIGHT G E Dietary factors concerned in erythropoiesis Blood 2. 112, 256 1947
- <sup>43</sup> CASSANO C. Anemie emolitiche Forme pure e forme combinate Bologna Cappelli Ed., 1930.
- <sup>44</sup> — AND BENEDETTI, G Il midollo osseo nella iperemolisi costituzionale Riv Clin Med 41 115 157 1940
- <sup>45</sup> —, AND — Iperemolisi costituzionale e disepatismo Rass Fisiopt cl e ter 17 Suppl 13 1945
- <sup>46</sup> CASTAONARI, G Intorno ad una particolare sindrome osteoporotica diffusa in un caso di anemia eritroblastica dell'infanzia Bull Sci Med Bologna 105 399 1933
- <sup>47</sup> — La sindrome radiologica dell'anemia mediterranea o anemia eritroblastica tipo Cooley Boll. Soc it Pediatr 3 513, 1934
- <sup>48</sup> CATALANO G La splenectomia nella sindrome di Cooley Policl (Sez Chir) 50 217, 1943
- <sup>49</sup> CATEL, W Ueber ein der Cooleyschen Anämie ähnliches Krankheitsbild Klin Wchnschr 21. II I, 848 1941
- <sup>50</sup> CHINI, V Sindrome di Cooley in adulto Boll Soc med chir Catania 6 282, 1938
- <sup>51</sup> — Su alcuni rapporti tra infezione malarica e sindromi tipo Cooley Haematologica 21 88 1939.
- <sup>52</sup> — Orientamenti moderni di clinica ematologica e loro rapporti con l'anemia mediterranea nei suoi riflessi storici e sociali Policl (Sez. Prat) 46 671 1939
- <sup>53</sup> — Risultati della splenectomia in alcune emopatie della serie rossa Med internaz. 47 219 1939.
- <sup>54</sup> — La sindrome di Cooley anemia mediterranea nell'adulto Rass Clin Scient 17 435 481 1939.
- <sup>55</sup> — Tentativi clinici di sistemazione nosologica dei cosiddetti itteri emolitici con resistenze globulari aumentate Gazz. med ital 104-105 205 1946
- <sup>56</sup> — Criteri di classificazione e sistemazione clinica delle sindromi ematologiche mediterranee Progr Med 4 1, 1948
- <sup>57</sup> — FERRANNINI A, AND MORATORE F Sul significato di un'azione favorente l'emolisi in vitro da parte del plasma di individui con cosiddetti itteri emolitici con aumentate resistenze eritrocitarie Boll Soc it Biol sper 16 712 1941

- <sup>17</sup> —, —, AND PONA, G. Indagini sulla familiarità dell'anemia mediterranea o morbo di Cooley. *Min Med* 30 I, 377, 1939
- <sup>18</sup> —, PATERNO, P., AND NICOTRA, A. Primi risultati di indagini radiologiche sullo scheletro di malarici cronici. *Boll Soc med chir Catania* 6 536, 1938
- <sup>19</sup> —, AND PEROSA, L. Di un particolare aspetto morfologico dei globuli rossi in anemie con aumentate resistenze eritrocitarie. *Boll Soc it Biol sper* 16 305, 1941
- <sup>20</sup> —, AND —. Sui rapporti tra ipocromia e sideremia nei cosiddetti itteri emolitici con iperesistenza globulare. *Boll Soc it Biol sper* 22 626 1946
- <sup>21</sup> CHITTI, G. Splenomegalia emolitica costituzionale con eritrocitosi ipocromica ed aumento delle resistenze globulari. *Rass Fisiopat cliv e ter* 13 335, 1941
- <sup>22</sup> CHOREMIS K., AND SPYLIPOPOULOS, J. De l'etiologie et du traitement de l'anémie type von Jaksch. *Cooley Latrika chronika* 83 1936 (abstr.)
- <sup>23</sup> —, AND —. Ueber die Actiologie und Therapie der Cooleyschen Anämie. *Jahrb Kinderh* 148 317, 1937
- <sup>24</sup> COLARIZI A. Ittero emolitico costituzionale con alterazioni ossee tipo Cooley. *ref Rif Med* 59 138 1943
- <sup>25</sup> —, AND BIDAUD, I. Anemie eritroblastiche splenomegaliche dell'infanzia. III) La malattia di Cooley. *Haematologica* 22 351 1940
- <sup>26</sup> — AND —. Anemie eritroblastiche splenomegaliche familiari. *Pavia, Tipogr Bianchi*, 1940
- <sup>27</sup> COOLEY T B. von Jaksch's anemia. *Am J Dis Child* 33 786, 1927
- <sup>28</sup> —. Likenesses and contrasts to the hemolytic anemias of childhood. *Am J Dis Child* 36 125 7 1928
- <sup>29</sup> —. Erythroblastic anemia. *Scripta med in oore* di R. Jemma 1 317, 1934
- <sup>30</sup> —. Hereditary factors in the blood dyscrasias. *Am J Dis Child* 62 8 1941
- <sup>31</sup> —. A severe type of hereditary anemia with elliptocytosis. *Am J M. Sc* 209 561, 1945
- <sup>32</sup> —, AND LEE, O P. A series of cases of anemia with splenomegaly and peculiar bone changes. *Tr Am Pediat Soc* 37 29 1925
- <sup>33</sup> —, AND —. Erythroblastic anemia. Additional comments. *Am J Dis Child* 43 707, 1932.
- <sup>34</sup> —, AND —. Role of erythrocyte fragmentation in the genesis of anemia. *J Pediat* 3 55 1933
- <sup>35</sup> —, WITWER E R. AND LEE, O P. Anemia in children with splenomegaly and peculiar bone changes. *Am J Dis Child* 34 347 1927
- <sup>36</sup> COOPER E L. Familial acholuric jaundice associated with bone changes. *Ann Int Med* 15 858, 1941
- <sup>37</sup> COTTINI G B. Sindrome anemica tipo Cooley in soggetto con lues congenita. *Dermosifil* 15 151, 1940.
- <sup>38</sup> CRAMER, B.—pers com to Thalheimer
- <sup>39</sup> CROSETTI, L., AND BAJARDI G. Presentazione di un caso familiare di splenomegalia emolitica con oote ematologiche iconosocite. *Atti R. Accad Fisiocr Siena* 3 148 1935
- <sup>40</sup> DALLA VOLTA A. Splenomegalia emolitica familiare eritemica (sindrome di Cooley). *Arch it. Pat e Cliv Med* 15 34, 1935
- <sup>41</sup> DAMESHEK, W. Target cell anemia. *Am J M Sc* 209 445 1940
- <sup>42</sup> —. Familial Mediterranean target-oval cell syndrome. *Am J M. Sc* 205 643, 1943
- <sup>43</sup> —. Hematology. *New England J Med* 237 434 480 1947
- <sup>44</sup> — AND SINGER K. Familial oonhemolytic jaundice. Constitutional hepatic dysfunction with indirect van den Bergh reaction. *Arch int Med* 67 259 1941
- <sup>45</sup> DEBRE R., LAMY M., MOZER, M., SÈR G. AND KAPLAN S. Maladie hémolytique chez deux jumeaux avec des déformations cranio-faciale et linguale. *Bull & Mém Soc Méd Hôp de Paris* 53 1465 1937
- <sup>46</sup> DHATAOUDE, R G. Erythroblastic anemia of Cooley (familial erythroblastic anemia) in an Indian boy. *Am J Dis Child* 67 290 1944
- <sup>47</sup> DI GUZIELMO L. Morfologia significato biologico significato clinico degli eritrociti a bersaglio. *Boll Soc it Biol sper* 20 743 1945
- <sup>48</sup> —. Sulle comparsa di eritrociti a bersaglio nei leucemici cronici in corso di roentgenterapia. *Boll Soc. it Biol sper* 22 1221 1946

- <sup>69</sup> DISCOMBE G AND WATKINSON G Atypical anemia with spherocytosis and target cells coexisting in the blood *Am J M Sc.* 213 153 1947
- <sup>70</sup> DOOLIOTTI G C Il substrato malarico quale fattore morbigeno *Acta med parav* 4 1 1943
- <sup>71</sup> DOMENICONI G Morbo di Cooley in adulto *Gazz. Osp.d e Clin* 63 307 1942.
- <sup>72</sup> DOMINICI G Ittero preepatico anemolitico *Lav Clin med Perugia* 15 1945
- <sup>73</sup> DONDI G Presentazione di un caso di anemia splenomegalica emolitica costituzionale con microcitosi e aumento di resistenza *Osped Magg Novara* 12 34 1935
- <sup>74</sup> — Sindromi emolitiche nell'infanzia *Varallo Sesia Atti graf De Grandi* 1937
- <sup>75</sup> ELIEL, L. D AND BAYLES T B Microcytic hypochromic anemia associated with splenomegaly and refractory to treatment *New England J Med* 225 134 1941 (Quoted by Wollman and Dickstein)
- <sup>76</sup> FABER H K. AND ROTH B M Mediterranean anemia (Cooley) in a Negro girl *J Pediatr* 21 610 1946
- <sup>77</sup> FAIRLEY N H *Tr Roy Soc Trop Med & Hyg* 34 173 1940 (quot by Schieber)
- <sup>78</sup> FANCONI G Die hyperchrome Elliptocyten anämie und ihre Differentialdiagnose gegenüber den Perniziösa und den Kugelmellanämie *Arch Kinderh* 117 1 1939
- <sup>79</sup> — Cooley oder Erythroblasten anämie *Schweiz med Wschr* 72 450 1942
- <sup>100</sup> FAWCZY A L. Cooley's anemia. Notes on 6 adult cases *J A M A* 132 955 1946
- <sup>101</sup> FEINGOLD B F AND CASE J T Roentgenologic skull changes in anemias of childhood (erythroblastic) Report of case & few notes on similar findings among skulls of Peruvian Indians *Am J Roentgenol* 29 194 1933
- <sup>102</sup> FERRANNINI A AND MURATORE F Azione dell'acido nicotinico e della sua amide sulla crisi sanguigna in alcune forme di anemia *Hæmatologica* 23 537 1941
- <sup>103</sup> — AND — Ulteriore contributo allo studio di un'azione favorevole l'emolisi in vitro del plasma di individui con cosiddetti itteri emolitici con aumentate resistenze eritrocitarie *Boll Soc Biol sper* 22 628 1946
- <sup>104</sup> FERRANNINI L Emodiostopatia di Cooley in adulto *Min Med* 33 II 57 1942.
- <sup>105</sup> FIESCHI A Die Mittelmeeranämie *Verh dtsh Ges inn Med* 52 283 1940
- <sup>106</sup> FORNARA P L'anemia di Cooley e l'itteroanemia con micropoichilocitosi ed aumento della resistenza (tipo Rietti-Greppi Micheli) *Atti 17 Congr it P.diat* 17 465 1940
- <sup>107</sup> — Considerazioni storiche e geografiche sull'anemia di Cooley *Gazz med ital* 104 105 1 1946
- <sup>108</sup> — Le nostre conoscenze attuali sull'ittero-anemia con micropoichilocitosi ed aumento della resistenza globulare tipo Rietti-Greppi Micheli *Gazz med ital* 106 69 1947
- <sup>109</sup> — AND DONDI G Splenomegalie eritremiche nell'infanzia con e senza aumentata emolisi *Atti Soc it Ematologia* 2 17 1936
- <sup>110</sup> FOSTER L. P Cooley's syndrome in a Chinese child *Am J Dis Child* 59 828 1940
- <sup>111</sup> FRANCAVIOLIA A Considerazioni sulla distribuzione in Italia del morbo di Cooley *Min Med* 31 1 6 1939
- <sup>112</sup> — Ricerche sul morbo di Cooley Studio clinico su alcuni casi di morbo di Cooley osservati in Puglia *Arch Sc Med* 64 395 1939
- <sup>113</sup> FREUDENBERG E Ueber die Cooley Anämie *Klin Wschr* 21 I 286 1942.
- <sup>114</sup> — AND ESSER M. Zur Pathogenese der Cooley-Anämie *ref Riv clin Pediatr* 40 509 1942.
- <sup>115</sup> FROLA G Azione dell'amide dell'acido nicotinico in vari casi di eritroblastosi cronica *Boll Soc it Biol sper* 17 365 1942.
- <sup>116</sup> FRONTALI G AND RASI F Itteri emolitici con aumentate e con ridotte resistenze eritrocitarie *Min Med* 28 II 349 1937
- <sup>117</sup> — AND — L'eritroblastosi e l'emolisi nella malattia di Cooley e di Di Guglielmo *Arch it Pediatr e Pueric* 7 259 1940
- <sup>118</sup> GALEONE A Il complesso achilia ipocromia ferrosensibilità nell'ittero emolitico costituzionale tipo Rietti-Greppi Micheli *Min Med* 35 I 261 1944
- <sup>119</sup> GÄNSLEN M Konstitutionelle hämolytische Anämie *Verh dtsh Ges inn Med* 52 238 1940
- <sup>120</sup> GARDNER L. B Cooley's erythroblastic anemia in two Chinese boys *J P.diat* 31 347 1947
- <sup>121</sup> GASTALDI F AND LEONARDI G Sopra un caso di icterus juvenilis intermittens con resistenze globulari aumentate *Riv clin Med* 47 459 1947
- <sup>122</sup> GATTO I Ricerche sul micelogramma nella malattia di Cooley *Sottim m d* 21 853 1947

- <sup>122</sup> — Ricerche sui familiari di bambini affetti da malattia di Cooley Soc Ital Ped - Sez Sicilia occid 14 XII 1947 Ref Medie infant 13 17 1942.
- <sup>124</sup> — Ricerche sui familiari di bambini affetti da malattia di Cooley Arch Ital Pediatr e Pueric 9 128 1942.
- <sup>125</sup> — Effetti della splenectomia nella malattia di Cooley Boll Soc It Biol sper 22 1009, 1946
- <sup>126</sup> — L'ipoevolotismo nella malattia di Cooley Giorn di Med 3 357 1946
- <sup>127</sup> — Talassemia (microcarterocitosi) Giorn di Med 4 287 1947
- <sup>128</sup> — Sulle ereditarietà della malattia di Cooley Min Med 39 1, 194 1948
- <sup>129</sup> GERBASI, M. L'anemia eritroblastica di Cooley in America e nei paesi mediterranei La Pediatr 45 553 1937
- <sup>130</sup> GOVAN C D Erythroblastic anemia of Cooley Observations on the effect of splenectomy performed in identical twins J Pediatr 29 504 1946
- <sup>131</sup> GRAZIOSI P. Gli uomini paleolitici della Grotta di S Teodoro (Messina) Firenze Spinelli ed 1947
- <sup>132</sup> GREENBLATT I J, COHN, T D AND DEUTSCH, H L. Mediterranean target-oval cell syndrome in an adult Chinese male Report of a case Ann Int Med 24 259, 1946
- <sup>133</sup> GREPPI, E. Ittero emolitico familiare con aumento della resistenza delle emazie Min Med 8 II 1, 1928
- <sup>134</sup> — L'iperglobulia microcitica (microcitosi vera) con aumento della resistenza osmotica massima come varietà ematologica nel quadro dell'ittero emolitico primitivo Min Med 26 I 409, 1935
- <sup>135</sup> — Sugli itteri emolitici con aumento della resistenza globulare Min Med 26 I, 409 1935
- <sup>136</sup> GRINNAN, A J Roentgenologic bone changes in sickle cell and erythroblastic anemia Report of nine cases Am J Roentgenol 34 297 1935
- <sup>137</sup> HADEN R L, AND EVANS F D Sick cell anemia in the white race Arch Int Med 60 133, 1937
- <sup>138</sup> HEILMEYER L. Blutkrankheiten Berlin J Springer 1942.
- <sup>139</sup> HITZROT J M. Unclassified type of splenomegaly in children Ann Surg 88 361, 1928
- <sup>140</sup> JIMENEZ DIAZ C, MORALES FLOREZUELO PANIAGUA E, RODA E AND ROMEO ORBROZZO J Anemia infantilis osteodisplastica (Enfermedad de Cooley) Rev Clin Españ. 6 222 1942
- <sup>141</sup> INGRAMA G. Iperemolisi costituzionale con microcitosi ed aumento delle resistenze globulari La Clin 4. 185 1938
- <sup>142</sup> INTROZZI, P. Anemia ipocromica splenomegalica emolitica con ovalocitosi (elliticitosi) poichilocitosi ed aumento delle resistenze osmotiche dei globuli rossi Splenectomia Haematologica (Arch.) 16 525, 1935
- <sup>143</sup> KATO K. AND DOWNEY, H. The hematology of erythroblastic anemia (type Cooley) Folia haematol 50 55, 1933
- <sup>144</sup> KOHLBACH W. Beitrag zur Klinik und Roentgendiagnostik der Cooleyschen Erythroblastenanämie Fortschr Roentgenstr 64. 281 1941
- <sup>145</sup> LEHNDORFF H. Die Erythroblastenanämie Erg inn Med 50 568 1936
- <sup>146</sup> LEONARDI G, CASULA G, TRUCCHI, O, CARCASSI U. La costituzione ematologica dei sardi con particolare riguardo ad alcune emopatie a sfondo emolitico Boll Soc It Biol sper 22 733 1946
- <sup>147</sup> LEONE A AND LUOAS E. Sulla anemia eritroblastica con osteoporosi (sindrome di Cooley) Clin Pediatr 18 463 1936
- <sup>148</sup> LICHTWITZ, L. Pathologie der Funktionen und Regulationen Leiden A W Sijr Uctgever 1936
- <sup>149</sup> MALAGUZZI VALERI C. Sul significato del sintoma ittero nel disordine ematologico mediterraneo Sett med (in press)
- <sup>150</sup> MALAMOS B AND DELIANNIS G. Beobachtungen über die Erythroblastenanämie des Mittelmeeres Verh dtsch Ges inn Med 52. 297 1940
- <sup>151</sup> MANAI A. La anemia splenica emolitica dei malarici cronici Giorn Clin Med 18 619 1937
- <sup>152</sup> MANDEVILLE F B. Roentgen ray findings in sickle cell anemia and Mediterranean anemia Radiology 15 72 1930
- <sup>153</sup> MARMONT A. Il midollo osseo ed i fattori mielopatici nella sindrome anemico-itterica di Rietti-Greppi Micheli Gazz med ital 106 88 1947
- <sup>154</sup> — AND BIANCHI V. Sull'importanza dei fattori corpuscolari nel determinismo della velocità di sedimentazione nella sindrome di Rietti-Greppi Micheli Boll Soc It Biol sper 1049 1946

- 154 —, AND — La frammeotaziooe globulare (schistomicrocitosi) oel midollo delle anemie tipo Rietti-Greppi Micheli Progr Med 3 606 1947
- 154 —, AND — Sopra uo ouovo fenomeno di frammeotaziooe degli eritrociti nella thalassemia. Boll Soc it Biol sper 22. 1047, 1946
- 157 —, AND — Importaoza dei fenomeoi eritroclastici, etc Pathologica 39. 9 1947
- 155 — AND — Ulteriori ricerche sui fattori corpuscolari nella velocità di sedimentazione nella sindrome Rietti-Greppi Micheli Boll Soc it. Biol sper 23 73 1947
- 159 — AND — L emoglobina nell anemia tipo Rietti-Greppi Micheli I) Proprietà cristallografiche. Boll Soc it Biol sper 23 1058 1947
- 160 —, AND — id II) Analisi spettrofotometriche Boll Soc it Biol sper 23 1061 1947
- 161 MATTEI, C Ulteriori osservazioni in tema di ittero emolitico a resistenze globulari aumentate ref Policl (Sez. Prat.) 54. 1197 1947
- 162 McINTOSH R AND WOOD C L. An inquiry into the genetic factor in Cooley's anemia Amer J Dis Child 64. 192 1942
- 160 MEULENGRACHT, E Icterus intermittens juvenilis (Chrooischer intermittierender juveniler Subicterus) Klio Wschr 28 I 118 1939
- 164 MICHELI F Splenomegalie emolitiche 35 Coogr Soc it Med 101 1929
- 163 — PENATI F AND MOMOLIANO LEVI G Anemia ipocromica splenomegalica con ellitocitosi poichilocitosi Atti Mem Soc Lomb Med 3 739 1935
- 166 — AND — Ulteriori ricerche sull anemia ipocromica splenomegalica con poichilocitosi Atti Soc ital Ematologia 1 5 1935
- 167 MILLS E S Thalassemia Arch 101 Med 62. 1004 1938
- 168 MOMOLIANO LEVI G In tema di oosografismo dell aemia emolitica costituzionale con poichilocitosi-ellitocitosi ipocromica Mio Med 38 I, 150 1947
- 169 MONCRIEFF A AND WHITBY L. E. H Cooley's anemia Laocet 2. 648 1934
- 170 MOORE S Booe changes in sickle cell anemia with note oo similar changes observed in skulls of ancient Mayan Indians J Missouri ed A 26 561 1929
- 171 MURATORE F Ricerche sulla familiarità del cosiddetto ittero emolitico con iperesistenza globulare Progr Med Suppl 3 14 1947
- 172 — L emolisi oelle eritropatie coo micro-ellitico-poichilocitosi iperesistente Rif Med (in press).
- 175 — AND CIRCELLA C Le granulazioni basofile nel sangue midollare e periferico nelle eritropatie tipo Rietti-Greppi Micheli Boll Soc it Biol sper 23 954 1947
- 174 —, AND DELL'AQUILA A La microcitosi oel cosiddetto ittero emolitico con iperesistenza globulare Boll Soc it Biol sper 22. 967, 1946
- 176 —, AND FIORE DONATI L. L ellitocitosi midollare e periferica oel cosiddetto ittero emolitico con iperesistenza globulare Boll Soc it Biol sper 22: 968 1946
- 178 NEEL J N AND VALENTINE W N The frequency of thalassemia Amer J M. Sc 209. 568 1945
- 177 — AND — Further studies oo the genetics of thalassemia Genetics 32. 38 1947
- 175 NERI F AND SOLIMINI, O Un caso di sindrome di Cooley in adulto Policl (Sez. Prat.) 52. 257 1945
- 178 NICOTRA A Indagini sulla familiarità dell anemia mediterranea Radiol med 26 720 1939
- 180 NITTS S AND SPILIOPOULOS G Similitary of erythroblastic anemia and chronic or congenital malaria Am J Dis Child 54. 60 1937
- 181 OLIVA G Sull ittero prepatito anemolitico Rif Med 59 1038 1945
- 182 ORTOLANI M. L anemia di Cooley in gemelle monocorie Il Lattante 7 323 375 1936
- 188 — L emoosteopatia di Cooley Min Med 28 I 191 1937
- 184 — Ueber die sogenannte Cooleysche Erkrankung Monatsch Kinderh k 71 174 1937
- 185 — AND CASTAGNARI G Contribinto allo studio della emo-osteopatia di Cooley Il Lattante 7 777 1936
- 184 — AND COSTA L. La splenectomia nel Morbo di Cooley Ref Rif Med 60 246 1947
- 187 — AND VALLINERI E Su trentadue casi di sindrome aemica di Cooley Bull Sci Med Bologna 112. 255 1940
- 188 PACHOLI R. La mielosi eritremica cronica tipo Cooley Clin Pediatr 22. 397 1940
- 189 PANOFF A Beitrag zur Cooley Anämie Mschr Kinderh k 73 184 1938

- <sup>100</sup> PANSINI G. Mieloblastosi familiare e diatesi emolitica nei consanguinei di una inferma affetta da ittero emolitico familiare Arch ist Bioch ital 3 283 1931
- <sup>101</sup> PARADISO F. Su di un caso di eritemia con ipertrofia del cuore Arch ital Pediatr e Pueric 1 405 1933
- <sup>102</sup> ———. Su alcuni casi di anemia con eritroblastosi ed alterazioni scheletriche a base prevalentemente osteoporotica Pediatr med Prat 9 519 1934
- <sup>103</sup> ———. Razza ereditarietà e infezione palustre nell'etiologia dell'anemia di Cooley Gazz med ital 104-105 22, 1946
- <sup>104</sup> PATRASSI, G. Konstitutionell familiäre und erworbene Cholämie Ztsch Klin Med 142 285, 1943
- <sup>105</sup> ——— AND TAOLIONI V. Circa l'esistenza della colemia costituzionale e familiare Atti R ist Veneto Sc Lett Arti 102 II, 73 1943
- <sup>106</sup> ——— and ———. Ulteriore contributo alla conoscenza degli stati colemici Acta med Patav 4 306 1943
- <sup>107</sup> ——— and ———. L'ittero emolitico costituzionale ellittopoichilolitico Acta med Patav 7 43 1946
- <sup>108</sup> PÉHU M., AND LERICHE, F. Sur l'anémie de Cooley ref K-Zbl inn Med 104 613 1940
- <sup>109</sup> ———, AND NOEL, R. Sur les érythroblastoses de l'enfance Arch méd des Enf 41 321 1938
- <sup>110</sup> PEROSA, L. I globuli rossi bersaglio e loro significato Haematologica 26 13 1945
- <sup>111</sup> ———. La ferremia e l'assorbimento del ferro nei cosiddetti itteri emolitici con resistenze globulari aumentate Boll Soc it. Biol sper 20 618, 1945
- <sup>112</sup> ——— AND PUTIONANO T. I globuli rossi a bersaglio nella malaria Boll Soc it Biol sper 22 641 1946
- <sup>113</sup> ——— AND VITERBO, F. Studio radiologico sulle lesioni ossee nell'ittero emolitico con iperesistenza globulare Boll Soc it. Biol sper 22 1063, 1946
- <sup>114</sup> PIERCE M. I. Erythroblastic anemia A clinical study Am J Dis Child 61 654 1941
- <sup>115</sup> PINCHERLE, M. Presentazione di due casi di sindrome di Cooley Bull Sci Med Bologna 106 169 1934, and 108 229 1936
- <sup>116</sup> ———, AND SCAOLIETTI O. Mielosi eritemica osteopatica Arch ital Pediatr e Pueric 6 101 1948
- <sup>117</sup> PONTONI L. Eritemia cronica familiare tipo Cooley Haematologica 18 377 1937
- <sup>118</sup> ———. L'eritropatia ellittopoichilolitica tipo Rietti-Greppi Micheli con microcitosi ipocromica ed aumento della resistenza osmotica Haematologica 24 479 1942.
- <sup>119</sup> PUTIONANO T., AND FIORE DONATI L. La resistenza emoglobinica nel Morbo di Cooley e forme affini Boll Soc it Biol sper 24 277, 1948
- <sup>120</sup> RACUONO A. Modificazioni del chimismo gastrico in bambini affetti da mielosi eritemiche ed eritroleucemie infantum Clin Pediatr 18 691 1936
- <sup>121</sup> RAVENNA F. AND CANNELLA C. Una forma nuova di grave anemia infantile associata ad osteoporosi diffusa Policl (Sez. Prat) 41 807 1943
- <sup>122</sup> RIETTI F. Sugli itteri emolitici primitivi Atti Accad Sci Med Ferrara 2 14 1924-25
- <sup>123</sup> ———. Le itterizie Bologna Cappelli, 1925
- <sup>124</sup> ———. Les icteres hémolytiques avec augmentation de la résistance globulaire Ann de Med 41 405 1937
- <sup>125</sup> ———. A proposito di itteri emolitici con resistenze globulari aumentate Min Med 29 II 95 1938
- <sup>126</sup> ———. Gli itteri emolitici con resistenze globulari aumentate nel quadro delle anemie emolitiche costituzionali Gazz med ital 104-105 222, 1946
- <sup>127</sup> ROHR K. Familiäre hämolytische hypochrome Anämie Helv Med Acta 10 31 1943
- <sup>128</sup> RUBIE J. Cooley's anemia Proc Roy Soc Med 41 42, 1948
- <sup>129</sup> RUNDLES R. W. AND FALLS H. F. Hereditary (Sex linked) anemia Am J M. Sc 211 641 1946
- <sup>130</sup> SANGIORGI M. Reperti di emazie a bersaglio in ratti parassitati Boll Soc. it Biol sper 17 483 1942.
- <sup>131</sup> SANGONE L. Splenomegalia emolitica costituzionale con microciti a vera ipocromica ed aumento delle resistenze globulari Arch Sc Med 65 1 1938
- <sup>132</sup> SANTILLANA A. Contribution à l'étude de l'anémie de von Jaksch-Lazet à forme familiale du type Cooley Arch Méd des Ent 41 356 1938
- <sup>133</sup> SARACOGULU K. Cooleysche Anämie in der Türkei Wien med Wchnschr 93 217 1943



- <sup>221</sup> SCHIEBER C Target-cell aemia Two cases in Bucharan Jews *Lancet* 2, 851 1945
- <sup>222</sup> SCOTT DOUGLAS R AND ROVERSI L L'anemia dei malarici acuti e cronici *Arch. Magaglio* 2, 15, 1947
- <sup>223</sup> SIONORELLI S Mielo i eritemica cronica tipo Cooley *Boll. Soc. med. chir. Catania* 5, 636 1947
- <sup>224</sup> SILVESTRONI E Anemia microcitica costituzionale *Polici (S-2 Med)* 51, 197 1944
- <sup>225</sup> ——— Moderni orientamenti sui fattori etiopatogenetici del Morbo di Cooley *Polici (S-2 Med)* 54, 1147 1947
- <sup>226</sup> ——— AND BIANCO I Prime osservazioni di resistenze globulari aumentate in soggetti sani e rapporti tra questi soggetti e i malati di cosiddetto ittero emolitico a resistenze globulari aumentate *Boll. e Atti Accad. med. Roma* 69, VI, 1943
- <sup>227</sup> ——— AND ——— La resistenza osmotica delle emazie nei soggetti normali *Pont. Accad. Scient., Commentationes* 8, 633 1944
- <sup>228</sup> ——— AND ——— Sull'esistenza nell'uomo di una particolare anomalia ematologica costituzionale *Boll. e Atti R. Accad. med. Roma* 70, 44 1944-45
- <sup>229</sup> ——— AND ——— Micro-drepanocito-anemia in un soggetto di razza bianca *Boll. e Atti Accad. med. Roma* 70, 347 1944-45
- <sup>230</sup> ——— AND ——— Dimostrazione nell'uomo di una particolare anomalia ematologica costituzionale *Polici (S-2 Med)* 52, 105 1945
- <sup>231</sup> ——— AND ——— Siogolare associazione di anemia microcitica costituzionale con drepanocitemia *Polici (S-2 Med)* 53, 265 1946
- <sup>232</sup> ——— AND ——— Ricerche sulla etiopatologia del M. di Cooley *Min. Med.* 37, II, 91 1946
- <sup>233</sup> ——— AND ——— Una particolare anomalia ematologica la microcitemia *Min. Med.* 37, II, 126 1946
- <sup>234</sup> ——— AND ——— Microcitemia *Ric. Morfol.* 22, 1946
- <sup>235</sup> ——— AND ——— Ricerche sui familiari dei malati di Morbo di Cooley *Ric. Morfol.* 22, 1946
- <sup>236</sup> ——— AND ——— Alcuni dati riassuntivi di ricerche cliniche, genetiche ed ematologiche sui rapporti tra anemia microcitica costituzionale e M. di Cooley *Min. Med.* 38, I, 587 1947
- <sup>237</sup> ——— AND ——— Nuove ricerche sulla trasmissione ereditaria della microcitemia *Polici (S-2 Med)* 54, 1359 1947
- <sup>238</sup> ——— AND ——— Alcune osservazioni sui familiari di malati di Morbo di Cooley e sulla frequenza dei portatori di microcitemia nel Ferrarese *Ric. Scient. e Ricostruz.* 17, V, 1947
- <sup>239</sup> ——— AND ——— Nuove ricerche sull'etiologia del Morbo di Cooley e prime osservazioni sulla frequenza della microcitemia nel Ferrarese *Min. Med.* 39, I, 205 1948
- <sup>240</sup> ——— AND GENTILI M Qualche osservazione antropologica sui portatori di microcitemia *Ric. Morfol.* 22, 1946
- <sup>241</sup> SIMONETTI C Studio radiologico delle lesioni ossee nel Morbo di Cooley *La Clin.* 6, 9, 1942
- <sup>242</sup> SIMONETTI CUZZA R Un caso di anemia eritroblastica tipo Cooley Splenectomy *Clin. Pediatr.* 11, 671 1936
- <sup>243</sup> SINGER K, MILLER E B AND DAMESHEK W Hematologic changes following splenectomy *Am. J. M. Sc.* 202, 171 1941
- <sup>244</sup> SMITH C. H. Familial blood studies in cases of Mediterranean (Cooley's) anemia *Am. J. Dis. Child.* 65, 681 1943
- <sup>245</sup> ——— Diagnosis of anemias in infancy and childhood *J. A. M. A.* 134, 992, 1947
- <sup>246</sup> SOVENA E AND AMMANNITI L Sulle indicazioni e sugli effetti immediati e tardivi della splenectomia *Atti e Mem. Soc. Rom. Chir.* 3, 17 1941
- <sup>247</sup> SPECIE R Considerazioni a proposito di un caso di ittero emolitico con resistenze globulari aumentate *Min. Med.* 32, I, 504 1941
- <sup>248</sup> SPYLIPOPOULOS G De la nature des lesions observees sur le squelette dans l'anémie type von Jaksch Cooley *Jatrika chronika* 1936 (abstr. greek)
- <sup>249</sup> ——— Welcher Natur sind die bei der Cooleyschen Anämie auftretenden Knochenveränderungen *Jahr. Kinderh.* 148, 329 1937
- <sup>250</sup> STILLMANN R G A study of von Jaksch's anemia *Am. J. M. Sc.* 153, 218 1917
- <sup>251</sup> SRANSKY E AND REGALA A C. Erythroblastic anemia following splenectomy *Am. J. Dis. Child.* 63, 859 1942

- 233 STRAUSS M. B. DALAND G. A. AND FOX H. J. Familial microcytic anemia. Observations on 6 cases of a blood disorder in an Italian family. *Am J M Sc* 201: 30 1941
- 234 TESTOLIN M. Splenomegalia emolitica costituzionale con aumento della resistenza globulare massima ovalocitosi e poichilocitosi. *Clin med ital* 67: 155 1936
- 235 — AND ANGELINI G. Contributo alla conoscenza di sindromi anemico-emolitiche splenomegaliche con aumento della resistenza globulare massima. *Osped Magg* 21: 275 1933
- 237 THALHEIMER E. J. MEZZETTI A. AND GERSHON-COHEN J. Cooley's anemia (Mediterranean anemia) with a report of a case in an adolescent. *J Pediatr* 14: 349 1939
- 238 TOSCANO F. Il quadro clinico della malattia di Ruetts-Greppi. *Micheli Gazz Med ital* 106: 83 1947
- 239 TRAMBUSTI B. Eritropoiesi ed amido-nicotinica. *Boll Soc ital Biol* 50: 17 363 1942
- 240 TRINÇAO C. Formas frustas da Doença de Cooley. *A Medic contempor* 43: 145, 1945
- 241 TROPP C. AND PENEFF L. Quantitative klinische Harnporphyrin untersuchungen. *Deutsch Arch klin Med* 180: 411 1937
- 242 USEGLIO G. AND DE MATTEIS F. Ulteriore contributo alla conoscenza dell'iperemolia familiare con aumento della resistenza osmotica dei globuli rossi. *Min Med* 25: I 861 1934
- 243 — AND MASSOBRIO E. Iperemolia costituzionale con aumento delle resistenze globulari. *Min Med* 25: I 603 1934
- 244 VALENTINE W. N. AND NEEL J. V. Hematologic and genetic study of the transmission of thalassemia. *Arch int Med* 74: 185 1944
- 245 VALLISNERI E. Su trenta casi di sindrome anemica di Cooley. *Policl infant* 8: 145 1940
- 246 VANNOTTI A. Porphyrin- und Porphyrinreaktionen. *Berlin J Spring* 1: 1937
- 247 VECCHIO F. Sulla resistenza di emoglobina alla daturazione alcalina negli ammalati di anemia di Cooley e nei loro familiari. *Progr Med* 4: 201 1948
- 248 VIGNERES N. W. AND SLOAN F. R. Mediterranean anemia. *J A.M.A.* 125: 352, 1944
- 249 WAKEFIELD E. G. DELLINGER S. C. AND CAMP J. D. A study of the osseous remains of the mound builders of Eastern Arkansas. *Am J M Sc* 193: 488 1937
- 250 WALLACE S. A. AND KILLINGSWORTH W. P. Sicklemia in the Mexican race. *Am J Dis Child* 50: 1208 1935
- 251 WANG CH. Y. AND KIM F. Y. Hemolytic anemia with erythroblastemia. A report of three cases seen in Chinese infants. *Ref K Zhil inn M d* 107: 290 1941
- 252 WHIPPLE G. H. AND BRAUER W. L. Racial or familial anemia of children associated with fundamental disturbance of bone and pigment metabolism. *Am J Dis Child* 44: 336 1932
- 253 — AND — Mediterranean diseases. Thalassemia. Associated pigment abnormalities simulating hemochromatosis. *J Pediatr* 9: 279 1936
- 254 WILLIAMS H. U. Human palaeopathology with some original observations on symmetrical osteoporosis in the skulls. *Arch Pathol* 7: 839 1929
- 255 WINTROBE M. M. MATTHEWS E. POLLAK R. AND DOBINS B. M. A familial hemopoietic disorder in Italian adolescents and adults. *J A.M.A.* 114: 1530 1940
- 256 WOLLMAN I. J. AND DICKSTEIN B. Changing concepts in Mediterranean (Cooley's) Anemia. *Am J M Sc* 212: 723 1946
- 257 WOLLSTEIN M. AND KREIDEL K. V. Familial hemolytic anemia of childhood. *Am J Dis Child* 59: 115 1930
- 258 ZUELZER W. W. Pathogenesis of anemia in infancy and childhood. *J A.M.A.* 134: 998 1947

# THE USE OF EXCHANGE TRANSFUSION FOR THE TREATMENT OF SEVERE ERYTHROBLASTOSIS DUE TO A-B SENSITIZATION, WITH OBSERVATIONS ON THE PATHOGENESIS OF THE DISEASE

By A S WIENER, M D , I B WEXLER, M D , AND J G HURST, A.B

IN PREVIOUS papers,<sup>1-3</sup> a method of treating erythroblastotic infants by exchange transfusion was described. In the cases reported, the disease had been produced by sensitization to the Rh<sub>0</sub> factor. Despite the publication of several well documented reports,<sup>4-15</sup> it is still not universally appreciated that typical erythroblastosis can also, though rarely, result from sensitization to the A and B factors. The purpose of this paper is to describe two unusual cases of severe erythroblastosis caused by sensitization to the A and B agglutinogens, respectively, which were treated successfully by exchange transfusion. In addition, observations will be described which are of significance with regard to the pathogenesis of such cases.

## MATERIALS AND METHODS

The most essential test in making an antenatal diagnosis of erythroblastosis is the titration of the antibodies in the maternal serum not only in cases caused by Rh sensitization but also in those produced by A-B sensitization. However, we have found that the methods which are optimal for titrating alpha and beta antibodies are not the same as those which have proved best in our hands for titrating Rh antibodies. This seems to be due in part to peculiarities of the agglutinogens and in part to peculiarities of the antibodies.

With regard to the agglutinogens or haptens these are presumably spaced at regular intervals about the periphery of the red cell and the following evidence is available to indicate that the A and B haptens are far more numerous than the Rh haptens.<sup>16-17</sup> (1) Red cells and stomata give much higher titers in inhibition tests with anti A or anti B sera than with anti Rh sera.<sup>17</sup> (2) Agglutinogens A and B are more potent antigens than Rh; however, this may also be explained on the basis of priming.<sup>18</sup> (3) Although properly performed Rh tests yield just as distinct reactions as ordinary A B tests, the clumps are much more fragile in the Rh tests indicating a smaller number of combining points. (4) While alpha and beta antibodies frequently produce complete lysis of sensitive cells in vitro as well as in vivo in vitro hemolysis is rarely seen in the Rh tests and then only in minimal amounts.<sup>19</sup> This again is presumably due to the smaller number of combining points on the red cell envelope. (5) The ability of strong univalent Rh antibodies to block Rh positive cells in contrast to univalent alpha and beta antibodies can be ascribed to the smaller number of specific Rh points to be coated.<sup>16</sup> These differences between the Rh and A B antigens could be expected to influence the optimal methods for their demonstration.

With regard to the nature of the antibodies the alpha and beta antibodies occur naturally while the Rh antibodies are practically always the result of active sensitization. The Rh antibodies are of two major types: agglutinins (bivalent antibodies) and blocking antibodies or glottinins (univalent antibodies). Univalent antibodies are relatively heat stable and traverse the placental barrier readily while bivalent antibodies are more heat labile and appear to be held by the intact placenta.<sup>9, 13, 18, 20, 21</sup> Thus as has been pointed out previously<sup>20-22</sup> it is the univalent antibody and not the bivalent antibody which crosses the placenta and produces fetal erythroblastosis. For demonstrating univalent Rh antibodies the block

From the Blood Transfusion Division and the Department of Pediatrics of the Jewish Hospital of Brooklyn, N. Y., and the Serological Laboratory of the Office of the Chief Medical Examiner of New York City.

Read before Society for the Study of the Blood, New York, February 17, 1949.

ing test<sup>22</sup> is the least sensitive method the plasma-conglutination test<sup>14</sup> is about five to twenty times as sensitive as the blocking test the albumin plasma test<sup>23</sup> is about two to four times as sensitive as the plasma-conglutination test while the acacia-conglutination test described below is about half as sensitive as the albumin plasma test \*

In the case of the alpha and beta antibodies the demonstration of specific glutinins (univalent antibodies) is considerably hampered by the practically universal presence of iso-agglutinins in human serum. Of course if the titers by the conglutination method are substantially higher than by the agglutination method the presence of the univalent antibodies may be inferred. However, in most cases the presence of univalent antibodies can be demonstrated † only by indirect means such as by their ability to traverse the placental barrier.<sup>12</sup> For demonstrating univalent alpha or beta antibodies the blocking test cannot be used at all. We do use the plasma-conglutination method but do not use the albumin plasma method because this gives no more sensitive results than the plasma method. The most satisfactory results for univalent alpha and beta antibodies were obtained by us with the acacia method to be described below. In our hands the antiglobulin method was not satisfactory for demonstrating univalent alpha and beta antibodies.

Before describing the actual techniques used a few general remarks are necessary, in view of the continued appearance of reports claiming extravagant titers as high as 82 million units.<sup>24</sup> With the methods used in our laboratory we have hardly ever obtained titers as high as or higher than ten thousand units. The excessive values reported in the literature are probably explained by faulty technique especially by carrying over when making serum dilutions. To avoid this pitfall we use separate tubes of saline in order to rinse the pipet thoroughly between dilutions. When extraordinarily high titers are obtained the titrations are repeated starting with an accurately prepared dilution of the serum e.g. 1 to 10 or 1 to 100, depending on the titer expected. Moreover a different pipet is used for each serum and each blood suspension. The titer is taken as the reciprocal of the highest dilution giving a one plus reaction. A typical titration should end somewhat so the following fashion ++ + + + + ± + +, + ± +, +, +, —. On the other hand if the titration ends so the following manner ++ + ± + + ±, ± + +, —, the technique is suspect and the titration should be repeated instead of taking the last one plus reaction as the end point. To consider hardly distinguishable reactions such as the trace or ± level as the end of a titration is certainly unsafe and this probably accounts for some of the false high titers reported in the literature.

The maximal agglutination titer which may reasonably be expected to exist can be determined by converting agglutinin titers into milligrams of antibody protein per cc. This has been done in detail by Barrett and Tripp<sup>25</sup> for bacterial agglutinins while Kabat and Bezer<sup>26</sup> have made similar determinations for alpha hemagglutinins by the method of precipitation using as the antigen a solution of purified A substance. For example these latter workers have found that an anti A serum with a titer of 512 units contains about 60 micrograms of antibody nitrogen per cc. This estimate appears to be conservative when one considers that red cells have about 8 to 16 times the diameter of bacterial cells so that a titer of 512 for red cells should correspond to a titer of 64 for bacterial antigens or about 500 micrograms of antibody nitrogen per cc. according to the work of Barrett and Tripp. Using the more conservative estimate table 1 was constructed. It will be seen that a titer of 16 000 units which is the maximum that has ever been encountered by this laboratory corresponds to about 12.4 mg. antibody per cc. or about 1.2 grams per cent. This would mean that about one half to one third of the serum globulin would have to be in form of antibody which is not an unreasonable concentration for highly immunized individuals. In the right half of the table are listed some of the higher titers examples of which have been reported in the literature. It will be seen that the highest titer claimed namely 82,000 000 units would imply a serum containing about 60 grams of protein in the form of antibody per cc.

For titrating alpha and beta antibodies fresh blood suspensions of groups A<sub>2</sub> and B were used routinely and in some cases also blood of subgroup A<sub>1</sub>. Group O blood was always included as a check on

\* Under ideal conditions the antiglobulin test of Coombs et al.<sup>14</sup> and Moreschi<sup>15</sup> gives titers about two to four times as high as the albumin plasma technique.

† Perhaps the method of fractionating Rh antibodies by dialysis devised by Witelsky et al.<sup>17</sup> could be applied successfully also to alpha and beta antibodies.

the specificity of the reactions. Saline suspensions were prepared washed once by centrifugation, and the sediment resuspended to make a 2 per cent suspension. Into each tube (7-8 mm inside diameter) in a series was placed a drop of a corresponding series of progressively doubled dilutions of the serum to be titrated e.g. undiluted  $\frac{1}{2}$ ,  $\frac{1}{4}$ ,  $\frac{1}{8}$ , etc. When titrating alpha antibodies a drop of suspension of blood of subgroup A<sub>2</sub> (or occasionally also A<sub>1</sub>) was added to each tube while group B cells were used for titrating beta antibodies. The mixtures were shaken and placed in a water bath or incubator at 37 C for one hour. The tubes were then gently shaken and the reactions read with the naked eye and checked under the low power of the microscope. The results of this reading constituted the saline or agglutination titer. To each tube was then added a drop of the acacia solution described below and the mixture shaken and re-incubated for another hour. The results of the reading after the second hour constituted the acacia-conglutination titer. A duplicate titration was set up in saline media at the beginning of the first hour, and after the cells had sedimented completely all of the supernatant fluid was removed from each tube with the aid of a fine capillary pipet. To each tube was then added a drop of oxalated plasma, group A plasma

TABLE 1—Correlation between Red Cell Agglutinin Titer and Antibody Concentration

Red cell agglutinin titer (units)	Approximate concentration of antibodies		Red cell agglutinin titer (units)	Approximate concentration of antibodies	
	mg N/cc	mg protein/cc		Gm N/cc.	Gm protein/cc.
1	00011	0007	20,000	0012	014
2	00012	0014	40,000	0045	018
4	00045	0028	80,000	009	056
8	0009	0056	160,000	019	12
16	0019	012	320,000	037	23
32	0037	023	640,000	075	47
64	0075	047	1,280,000	15	94
128	015	094	2,560,000	3	19
256	03	19	5,120,000	6	38
512	06	38	10,240,000	12	75
1,024	12	76	20,480,000	24	150
2,048	24	15	40,960,000	48	300
4,096	48	30	81,920,000	96	600
8,192	96	60			
16,384	19	120			

being used for the alpha titrations and group B plasma for the beta titrations. The cells were resuspended the mixtures incubated for a second hour and the reactions read. The results of this test were designated the plasma-coagglutination titer.

The solution used for the acacia-conglutination titration was prepared by dissolving 10 grams of gum acacia and 1 gram of dibasic sodium phosphate ( $\text{Na}_2\text{HPO}_4$ ) in 90 cc of distilled water and sterilizing at once in the autoclave at 10 pounds of pressure for ten minutes.<sup>21</sup> The resulting opalescent solution is usable for a long time if kept sterile. It is important not to use too much heat when sterilizing or else the solution will not be active. Presumably excessive heating splits the acacia as indicated by the observation that such preparations are less viscous and more transparent instead of opalescent.

### CASE REPORTS

**Case 1.** The mother of this patient was referred to us because in routine antenatal tests she had been found to be Rh negative and her husband Rh positive. Her first pregnancy had terminated one year previously with the birth of a 7 pound 4 ounce infant who was delivered two weeks postmaturely after a labor of about eighteen hours. This child exhibited neither jaundice nor anemia and is now alive and well. The mother had never received any blood or plasma injections. The mother when first seen was entering the second trimester of her second pregnancy.

Grouping and Rh Hr tests done on the prospective parents and the first child gave the results shown in table 2. Also included in table 2 are the results of the saliva tests done on the family at a subsequent date.

Tests for Rh antibodies done on the mother's serum at this and subsequent examinations gave negative results by the agglutination method and the albumin plasma and acacia conglutination technics so that it was clear that she was not sensitized to the Rh factor. In view of the incompatibility to the major blood groups, the alpha and beta antibodies in the mother's serum were titrated. The results of these tests are shown in table 3 which lists the titers of alpha and beta antibodies as determined by the agglutination method and by the plasma and acacia-conglutination methods at various intervals throughout the remainder of her pregnancy. In view of the extraordinarily high anti B titer (average about 2-4 thousand units) we felt that she was carrying a group B fetus (cf Polayes et al<sup>11a</sup>) who might well prove to be erythroblastotic but on the basis of B sensitization rather than Rh sensitization.

The mother went into spontaneous labor at term and delivered a 7 pound 4 ounce male infant who appeared to be normal except for some cyanosis of the extremities and very mild jaundice. Examination

TABLE 2.—Grouping and Rh Hr Tests in Case 1

Blood of	Group	Rh-Hr Type		Saliva
		Phenotype	Genotype	
Father	B	Rh <sub>+</sub>	R <sup>0</sup> R <sup>0</sup> or R <sup>0</sup> r	Secretor
Mother	O	rh	rr	
Daughter (1st child)	B	Rh	R <sup>0</sup> r	Secretor

TABLE 3.—Results of Antenatal Antibody Titrations on Maternal Serum in Case 1

Time of Test		Rh anti bodies	Titer for A <sub>1</sub> B cells in			Titer for B <sub>1</sub> r cells in		
Date	Week of gestation		Saline	Plasma	Acacia	Saline	Plasma	Acacia
6-2-47	13	None	100	100		5,000	4,000	
7-9-47	18	None	30	60		1,800	1,800	
8-10-47	24	None	48	48		4,000	10,000	
9-21-47	28	None	60	50		1,920	>4,000	
11-10-47	36	None	50	300	800	2,000	2,000	8,000
12-3-47	39	None	40	30	120	1,600	1,600	3,200

of the cord blood revealed that the hemoglobin concentration was 11 grams per cent and the white blood cell count 31,450 per cu. mm. There were 22 nucleated red blood cells per 100 white blood cells on the smear. The icterus index was 36 units. The infant was found to belong to group B type MN type Rh<sub>+</sub>. Despite the fact that the baby belonged to group B it was possible to demonstrate free beta antibodies in addition to alpha antibodies in its serum by the slide technic—an unprecedented finding up to that time in our own experience.

In view of these observations there was no doubt that the baby had erythroblastosis fetalis but due to B sensitization rather than to Rh sensitization in accordance with the prediction. It was therefore decided to treat the infant with exchange transfusion using blood of group O. One group O Rh negative and one group O Rh positive donor were selected each of whom had low titers of anti B agglutinins in his serum. The Rh positive donor was selected as a control in order further to test the conclusion that Rh sensitization had nothing to do with the patient's illness. By the time the preparations for the procedure were completed the jaundice had increased markedly (the icterus index determined subsequently proved to be 64 units by the acetone method as against 36 units at birth) and several petechiae had appeared on the face.

Five hundred cc. of blood were drawn from each donor into 60 cc. of citrate solution and this fresh

blood mixed with 10 cc. of a solution of A and B group substances\* was used for the transfusion. A total of 920 cc. of the citrated blood was introduced into the saphenous vein at the ankle while 820 cc. of blood were removed from the radial artery at the wrist over a period of 2 hours and 40 minutes. Heparin was used in the usual way to facilitate the bleeding, and calcium gluconate injected in fractional doses to counteract the effect of the citrate injected. The baby withstood the procedure well.

The following morning the baby's color was good and there was hardly any noticeable jaundice. The blood count at that time was hemoglobin concentration, 15.9 grams per 100 cc., and white blood cell count 12,400 per cu. mm. Two days after the transfusion progress seemed to be satisfactory except that edema of the legs and dorsum of the feet was noted. This subsided within twenty-four hours. On the third day the blood count was as follows: hemoglobin concentration 17.4 grams per 100 cc., red blood count 5.8 million per cu. mm., white blood count 11,700 per cu. mm., polymorphonuclear leukocytes 77 (24 band forms), myelocytes 5, monocytes 6, eosinophiles 12, non-nucleated red blood cells were seen on the smear. (As has been pointed out previously<sup>2</sup> the eosinophiles are regarded as due to the presence of an antigen-antibody complex in the infant's body.) The remainder of the baby's stay in the hospital was uneventful. A blood count done on the sixth day showed that the hemoglobin concentration was 14.1 grams per 100 cc., the red blood count 4.8 million per cu. mm. and the white blood count, 13,250 per cu. mm. Differential count showed polymorphonuclear leukocytes 33 (8 band forms), myelocytes 2, eosinophiles 17, lymphocytes 44, and monocytes 6. On the eighth day the percentage of eosinophiles had fallen to 2, and on the eleventh day the infant was discharged.

When the baby arrived home diarrhea developed with loss of weight down to 5 pounds 14 ounces at the end of three days. The diarrhea was treated at another hospital with starvation, parenteral fluids and blood transfusion. It is of interest to note that at that hospital the baby was typed as group O and that the transfusionist refused to accept our word that the infant really belonged to group B. As a result the infant continued to receive group O blood. It was not until the baby was seven weeks old that the diarrhea was under control and the baby regained its birth weight of 7 pounds.

The infant's subsequent physical and mental development has been normal. He held his head up at the age of 3 months and sat at 6 months. When seen again at the age of 10 months he weighed 23 pounds, was 30 inches long and was beginning to walk. His general demeanor was bright and he was responsive to his environment.

Of particular interest were the comparative studies of the antibody content of the maternal and infant's serum at birth, and the subsequent course of the antibody titers in the baby's serum after delivery. As shown in table 4, the baby not only had free alpha antibodies in his serum but also had substantial amounts of free beta antibodies despite the fact that he belonged to group B. Ordinarily, any beta antibodies passing into a group B fetus would be expected to be absorbed by the baby's cells and body fluids, leaving no free antibodies in the plasma. Only when a large excess of antibodies filters into the fetal circulation can free incompatible antibodies be expected to be demonstrable in the baby's serum, just as occurs in severe erythroblastosis due to Rh sensitization. Judging from the amount of univalent alpha antibodies present in the baby's serum, namely, about 100 units, one could estimate that  $\frac{1}{3}$  to  $\frac{1}{2}$  of the maternal alpha antibody titer in plasma or acacia medium represents univalent antibody. If we assume the same proportion for the maternal beta antibodies, one could postulate a titer of about 500 to 1,000 units of beta univalent antibodies capable of filtering through the placenta, so that the presence of 80 units of free beta antibody in the baby's serum does not seem excessive under these conditions.

That these babies are not killed outright by the incompatible antibodies may

\* Obtained from Sharpe and Dohme.

seem remarkable at first sight. However, at least two protective mechanisms\* appear to exist which prevent such an outcome, namely, the low concentration of *conglutinin* in the fetal plasma<sup>16-22</sup> and the differences in sensitivity between the red cells of the newborn and those of the adult.<sup>23</sup> Univalent antibodies coat the cells of the infant, but without the aid of the third component, *conglutinin*, cannot clump them, so that until the process of maturation provides sufficient *conglutinin* the disease is held in abeyance. The lower sensitivity of the fetal red cells serves to work in the same direction. The titrations reported here were carried out with red cells obtained from *adults* and not with blood from infants, so that the titers are actually somewhat misleading. In fact, tests of the baby's plasma against its own red cells did not produce clumping even in the presence of adult plasma or *acacia*, indicating that the unabsorbed free beta antibody in its plasma represented

TABLE 4.—Comparison of Antibody Titers in Maternal Serum and Infant's Serum at Birth and after Birth (Case 1)

Serum	Titer for A <sub>rh</sub> cells			Titer for A <sub>rh</sub> cells			Titer for B <sub>rh</sub> cells			Icterus Index
	Saline	Plasma	Acacia	Saline	Plasma	Acacia	Saline	Plasma	Acacia	
Mother at delivery	160	240	480	50	100	240	2,560	4,000	5,000	—
Baby's cord serum	3*	40	96	3*	30	80	3*	16	80	36
Baby's serum before transfusion	0	12	160	0	0	16	1½*	9	24	64
Baby's serum after transfusion	1½*	30	196	0	6	20	1½*	6	16	24
Baby's serum at 1 week of age	1½*	20	18	1*	3	3	0	0	3	24

\* The reactions indicated by asterisks though occurring in saline media, are probably due to *conglutination* rather than *agglutination* similar to the phenomenon sometimes noted when titrating *trivalent* Rh antibodies in saline media.

that component incapable of reacting with its own group B red cells. It will be noticed that the antibodies disappeared relatively rapidly from the baby's circulation, as indicated by the much lower titers found at the age of one week, at which time the excessive numbers of *cosinophiles* also disappeared.

When the baby was born, 10 cc of a solution of group substances had been introduced into his umbilical vessels. This had no appreciable effect, however, in arresting the progress of the disease, so that the exchange transfusion was carried out. It will be seen, moreover, that despite the injection of group substances there was only a partial reduction in the antibody titers between the time the baby was born and the time the transfusion was started. Tests done later on showed that the baby was a *secretor*. The observations in this case as well as in others (cf. page 1028), therefore, indicate that *Levine's* assumption<sup>25</sup> that maternal alpha and beta antibodies affect only babies who are *nonsecretors* is not correct. This phase of

\* The possible existence of other protective factors in the body of the fetus and newborn may prove a profitable field for investigation.<sup>1-24</sup>



the pathogenesis of erythroblastosis fetalis caused by A and B sensitization will be discussed in greater detail later on

*Case 2* The mother of this baby was first seen by us in October 1946 when she was referred for grouping and Rh Hr tests because of the following history. She had never received any injection of blood or plasma and had had only a single pregnancy which terminated on December 6, 1943 with the birth of a male child. This first baby became jaundiced 24 hours after birth and was given a blood transfusion when he was a few days old. He was still jaundiced at the age of nine days when he was discharged from the hospital and the parents remarked that the jaundice persisted for several weeks at home. This child is alive and well and has no sequelae of his neonatal illness. Grouping and Rh Hr tests done on the family at the time of the first visit gave the results shown in table 5.

These results proved that the Rh factor had nothing to do with the baby's illness and suggest that sensitization to the agglutinin A might be the cause. Evidence supporting this surmise was obtained by titrating the maternal alpha and beta antibodies when it was found that the titer of alpha antibodies

TABLE 5—Grouping and Rh-Hr Tests in Case 2

Blood of	Group and subgroup	Rh Hr type		Saliva
		Phenotype	Genotype	
Father	A <sub>1</sub>	Rh <sub>1</sub> Rh <sub>2</sub>	R <sup>1</sup> R <sup>2</sup> R <sup>1</sup> r' r'R <sup>2</sup> R <sub>2</sub> etc.	Secretor
Mother	O	Rh <sub>1</sub> Rh <sub>1</sub>	R <sup>1</sup> R <sup>1</sup> or R <sup>1</sup> r'	
1st child	A <sub>1</sub>	Rh <sub>1</sub> Rh <sub>1</sub>	R <sup>1</sup> R <sup>1</sup> or R <sup>1</sup> r'	Secretor

TABLE 6—Results of Alpha and Beta Titrations on the Maternal Serum in Case 2 Before and During Second Pregnancy

Week of gestation	Titer against A <sub>2</sub> cells in			Titer against B cells in		
	Saline	Plasma	Acacia	Saline	Plasma	Acacia
Oct. 1946 (not pregnant)	200	1,280	—	60	100	—
June 22, 1948 (27 weeks)	24	48	400	24	48	160
August 11, 1948 (35 weeks)	56	150	300	20	40	100

was considerably elevated especially by the coagulation method (cf table 6) despite the long interval of almost three years since the child had been born.

The mother became pregnant for the second time in 1947 but this pregnancy terminated in a spontaneous abortion at two months. She became pregnant for the third time in 1948 and was retested in June 1948 when her pregnancy had progressed to 27 weeks. At that time titrations of the alpha and beta antibodies showed that their titers had fallen considerably (cf table 6). One month later there was some increase in titer but not to an alarming degree.

The patient a female infant weighing 8 pounds 13 ounces was delivered spontaneously at term. Jaundice was present at birth although the baby appeared to be normal otherwise. A blood count revealed a hemoglobin concentration of 16.9 grams per 100 cc, an uncorrected white blood cell count of 21,500 per cu mm, 44 per cent polymorphonuclear leukocytes of which 14 were band forms, 50 lymphocytes, 6 monocytes and about 100 nucleated red blood cells per 100 white blood cells on the smear. Examination of the cord blood showed that the infant belonged to group A and that the cord serum had an icterus index of 44 units by the acetone method. Moreover while the baby's cells formed a smooth suspension in saline they clumped spontaneously when suspended in plasma or acacia. Although the baby belonged to group A free alpha antibodies could be demonstrated in her serum by the coagulation method. In view of these findings a diagnosis of erythroblastosis due to sensitization to the agglutinin A was made and 10 cc of a solution of A and B group substance were administered intravenously.

During the forty-eight hours following birth there was a slight drop in hemoglobin to 13.5 grams per 100 cc and the blood smear then showed only 17 nucleated red blood cells per 100 w b c. However, the jaundice increased markedly so that the icterus index reached 12.8 units by the acetone method. A second injection of group substance was given, and in view of the progressive nature of the disease it was decided to do an exchange transfusion using group O donors.

Accordingly 500 cc of blood were drawn from each of the two group O Rh positive donors into 60 cc of sodium citrate solution. To each bottle of blood were added 10 cc of a solution of A and B group substances. Nine hundred cc of citrated blood were introduced into the baby by way of the saphenous vein at the ankle while 800 cc were removed through the radial artery over a period of two hours. Heparin was used to facilitate the bleeding and calcium gluconate was injected in fractional doses to counteract the effect of the injected citrate. The baby withstood the procedure well. By the following morning the jaundice had appreciably diminished. However six hours following the transfusion, the baby's temperature rose to 103 F and remained elevated for 48 hours. No abnormal physical findings were detected to account for the rise in temperature. The patient was given routine prophylactic penicillin injections, 20,000 units every three hours, as is done with all infants following exchange transfusion but because of the rise in temperature this was continued for three days instead of the usual twenty-four hours.

By the time the baby was one week old there was no further evidence of erythroblastosis. The icterus index had fallen to 4 units and the patient was discharged from the hospital on the eighth day of life. At the age of 12 days the baby was well. The hemoglobin concentration was 13.4 grams per 100 cc, the red blood cell count was 4.51 million per cu mm and the white blood cell count 8,200 per cu mm. Polymorphonuclear leukocytes 31 (3 band forms), lymphocytes 35, monocytes 10, eosinophiles 4, only 1 late normoblast per 10 w b c on the blood smear. The icterus index was 4 units and the baby's blood reacted as group O, there being no trace of its own group A blood demonstrable. The baby was last seen at the age of four months when she was perfectly well and exhibited no sequelae of her illness.

That this baby was so severely affected came somewhat as a surprise, in view of the relatively low titers obtained in the antenatal titrations of the alpha antibodies in the maternal serum. A test made on the mother on the day of delivery showed, however, that there had been a substantial increase in the alpha antibody titer of the maternal serum (cf table 7), particularly by the agglutination technic, and this explained the findings in the baby. In this case as in the previous one, the injection of A and B group substances had no noticeable effect in arresting the progress of the disease, and there was only a rather disappointing drop if any, in the titer of univalent alpha antibodies in the baby's serum (cf table 7). Therefore, exchange transfusion was resorted to, and the clinical improvement which followed was so prompt and dramatic that there can be hardly any doubt that the baby's rapid recovery was due to the transfusion.

The alpha antibody titers were followed in the baby, and in tests made nine days after birth it was found that the antibodies had almost entirely disappeared (cf table 7). On the other hand, tests done on the mother's serum showed no significant change in titer. It is important to point out that at the end of the transfusion the baby still had significant amounts of alpha antibodies in its plasma. Despite this, her clinical condition improved, indicating that the alpha antibodies *per se* are not toxic to a group A baby in the absence of group A cells with which they can combine. Thus, the antibodies apparently produce organic damage only through the blood stream, presumably by clumping the cells and blocking the circulation, and contrary to the assertion of some workers, do not react directly on the organ cells<sup>18</sup> by virtue of the specific antigens that such cells are supposed to contain.

## NORMAL BABIES AND THEIR MOTHERS

The two cases which have just been presented raise the question as to what findings are obtained when group O mothers have normal group A and group B children, i e., without clinical jaundice or anemia. If we are to ascribe significance to the high antibody titers obtained in these two cases, it is necessary to demonstrate that the titers in normal cases are appreciably lower.

Until recently, no studies had been made concerning univalent alpha and beta antibodies in normal individuals, in fact, until the Rh blocking antibody was found it was not appreciated that such antibodies existed at all. It was generally accepted that the natural alpha and beta antibodies were agglutinins reacting

TABLE 7—Comparison of the Titers of Maternal Serum and Infant's Serum at Birth and Before and After Exchange Transfusion (Case 2)

Serum	Titer against A <sub>1</sub> cells in			Titer against A <sub>2</sub> cells			Titer against B cells in			Icterus Index
	Saline	Plasma	Acacia	Saline	Plasma	Acacia	Saline	Plasma	Acacia	
Mother at delivery	140	650	6,400	140	440	4,800	60	140	400	—
Baby's cord serum	0	2	40	0	0	16	1½*	15	60	44
Baby's serum before exchange transfusion (age 48 hours)	0	6	12	0	3	3	1½*	3	24	128
Serum of 1st donor	—	—	—	5	48	40	8	64	64	—
Serum of 2nd donor	—	—	—	6	10	20	6	80	80	—
Baby after exchange transfusion	0	10	24	0	10	20	0	20	20	48
Mother 1 wk. after delivery	—	—	—	240	320	2,500	40	80	160	—
Baby's serum at 1 wk. of age	—	—	—	0	½	1½	1*	1	6	4

\* These reactions probably represent conglutination rather than agglutination (cf. footnote to table 4)

better at refrigerator than at body temperature.<sup>37</sup> These alpha and beta agglutinins were supposed to develop spontaneously in the serum as a sort of maturation process of the normal serum globulins, and were not considered to be the result of immunization. Individuals of all four blood groups were believed to produce both alpha and beta agglutinins, but the incompatible antibodies were supposed to be neutralized as quickly as they formed. That the normally maturing globulin should have alpha and beta specificity did not appear entirely surprising, when one considered that globulins having such specificity could also be obtained from bean extracts.<sup>38</sup> Therefore, the idea seemed reasonable that the natural alpha and beta agglutinins are normal serum globulins of large molecular size, and therefore incapable of traversing the placental barrier and doing harm to the baby should it belong to any incompatible group. Consequently, when a baby of an incompatible group developed jaundice and anemia, this was ascribed<sup>13</sup> to the presence in the maternal serum of *univalent* alpha and beta antibodies, presumably the

result of iso-immunization. These concepts have to be modified somewhat in the light of findings which are now to be presented.

In table 8 are listed a random series of cases in which mothers had normal babies belonging to compatible blood groups. It will be seen that the titers of the alpha and beta antibodies in these mothers are in an entirely different range from the titers exhibited by the mothers of the two babies treated by exchange transfusion, and as would be expected, the icterus indices of the cord serums were also within the normal range for newborn infants. The ratios between the titers of the alpha and beta antibodies in the maternal and infant's serums vary according to the method of titration used. In only a minority of instances was it possible to demonstrate the presence of antibodies in the infant's serum by the agglutination method (in saline media), and then in such low titer as to suggest that these reactions were actually due to conglutination\* rather than agglutination. By the conglutination method, in both plasma and acacia media, closer agreement is found between the maternal and infant's titers, and in some instances the titers are even equal. According to these findings, as well as evidence presented elsewhere,<sup>20</sup> it would appear that the antibodies present in the infant's serum at birth are always glutinins (univalent antibodies) derived passively from the mother, and that the maternal alpha and beta antibodies are usually mixtures of univalent antibodies (glutinins) and bivalent antibodies (agglutinins) rather than pure agglutinins. The proportion of glutinins to agglutinins seems to vary from person to person. In occasional instances, the alpha and beta antibodies are not demonstrable, or only barely so, in the baby's serum despite a substantial titer of antibodies in the maternal serum, presumably because the latter are essentially pure agglutinins which cannot traverse the placental barrier.

In view of these findings it is evident that the concept that natural alpha and beta antibodies are always pure agglutinins must be discarded, since a large proportion of normal individuals possess univalent alpha and beta antibodies as well. If we wish to adhere to the concept that univalent antibodies are produced as the result only of active sensitization, the question would arise as to how such sensitization could be produced against the A and B factors. In the course of a life-time it is not difficult to imagine how A and B factors could be introduced into the body through food, or infection, or vaccines administered for protective immunization, in view of the ubiquitous nature of A-like and B-like antigens.<sup>12, 27</sup>

Since many normal individuals possess univalent alpha and beta antibodies

\* The assertion that the antibodies in the infant's serum at birth are always glutinins (univalent antibodies) may seem to be contradicted by the numerous studies in the literature including some of our own earlier papers<sup>22, 40</sup> dealing with alpha and beta agglutinins in the serum of newborns. However, before the Rh blocking antibodies were found it was not appreciated that univalent alpha and beta antibodies existed and so any clumping of A or B specificity was assumed to be agglutination and to be produced by agglutinins. In retrospect there seems hardly any doubt that in our earlier studies we were actually dealing with conglutination and not with agglutination. Under favorable conditions conglutination sometimes occurs in saline media. For example, while American workers found Rh agglutinins 10 less than half the mothers of erythroblastotic babies, British workers reported that about 95 per cent of the sera from such women contained Rh agglutinins. We now know<sup>22</sup> that the British workers were dealing with conglutination which occurred in their tests despite the use of saline blood suspensions.

TABLE 8—Comparison of Titers in Maternal and Infant's Serums in Cases in which the Blood Groups are Compatible

Case No	Titration medium	Anti A titers (units)		Anti B titers (units)		Icterus index (cord serum)
		Mother	Baby	Mother	Baby	
Mother group O baby group O						
1	Saline	24	0	12	0	4
	Plasma	80	12	14	12	
	Acacia	80	24	70	64	
2	Saline	24	(1)*	32	(1)	12
	Plasma	40	6	21	6	
	Acacia	64	12	50	40	
3	Saline	6	(1½)	40	(1½)	8
	Plasma	10	3	40	6	
	Acacia	24	12	70	20	
4	Saline	80	0	120	(1½)	—
	Plasma	160	1	240	10	
	Acacia	240	2	480	16	
5	Saline	8	(1½)	16	(1½)	8
	Plasma	20	8	40	8	
	Acacia	90	32	48	24	
6	Saline	10	0	80	(3)	12
	Plasma	12	0	92	24	
	Acacia	20	5	256	40	
7	Saline	11	(3)	22	(1½)	14
	Plasma	20	0	36	0	
	Acacia	120	16	52	16	
8	Saline	20	(1½)	12	(1½)	12
	Plasma	24	12	20	22	
	Acacia	80	40	60	32	
9	Saline	12	0	12	0	12
	Plasma	10	0	20	0	
	Acacia	32	10	12	4	
Mother group A baby group A						
10	Saline	0	0	12	0	8
	Plasma	0	0	—	—	
	Acacia	0	0	40	1½	
11	Saline	0	0	4	(1)	10
	Plasma	0	0	12	2	
	Acacia	0	0	12	2	
12	Saline	0	0	6	0	10
	Plasma	0	0	22	4	
	Acacia	0	0	22	10	
13	Saline	0	0	20	0	4
	Plasma	0	0	40	0	
	Acacia	0	0	22	1½	
14	Saline	0	0	32	0	12
	Plasma	0	0	64	1	
	Acacia	0	0	48	6	
Mother group B baby group B						
15	Saline	60	0	0	0	—
	Plasma	15	—	0	0	
	Acacia	60	1½	0	0	
16	Saline	64	0	0	0	10
	Plasma	24	0	0	0	
	Acacia	80	3	0	0	

\* Figures in parentheses probably represent agglutination despite the occurrence of the reactions in saline media (cf footnote to table 4)

capable of traversing the placental barrier, it might be expected that when the mother carries a fetus of an incompatible blood group hemolysis of the fetus's cells should be inevitable. As already pointed out, however, the principal protective mechanism in the baby seems to be the relatively low sensitivity of the A and B agglutinogens of the newborn's red cells, so that only when the univalent

TABLE 9.—*Comparison of Titers in Maternal and Infant's Serum in Cases Where the Blood Groups Are Incompatible but in which the Infant is Normal*

Case no	Titration medium	Anti A titers (units)		Anti B titers (units)		Icterus index of cord serum
		Mother	Baby	Mother	Baby	
Mother group O, baby group A						
1	Saline	6	0	2	0	8
	Plasma	6	0	3	tr	
	Acacia	6	1½	12	2	
2	Saline	1½	0	16	(1)*	8
	Plasma	1½	tr	20	½	
	Acacia	8	1½	24	6	
3	Saline	32	0	5	0	—
	Plasma	10	0	3	½	
	Acacia	40	0	12	3	
4	Saline	6	0	40	(2)	—
	Plasma	2	0	40	3	
	Acacia	6	0	64	12	
5	Saline	20	0	64	0	—
	Plasma	48	0	80	3	
	Acacia	80	½	128	5	
6	Saline	80	0	60	(1½)	12
	Plasma	160	1	240	10	
	Acacia	240	2	430	10	
Mother group O, baby group B						
7	Saline	10	(1½)	4	0	10
	Plasma	12	4	10	0	
	Acacia	40	24	16	1½	
Mother group A, baby group B						
8	Saline	0	0	96	0	—
	Plasma	0	0	400	0	
	Acacia	0	0	800	1½	

\* Figures in parenthesis probably represent conglutination despite the occurrence of the reactions in saline media (cf footnote to table 4)

alpha and beta antibody titers of the mother reach extraordinarily high levels does harm to the baby result. A remarkably similar situation exists in cattle as has been recently found by Yeas.<sup>41</sup>

For purposes of comparison we have listed a series of antibody values obtained in cases in which mothers had normal babies of incompatible blood groups (cf table 9). It will be noted that the titers were within normal limits in every

instance except one (case 8), in which a group B baby of an O mother was apparently unaffected despite a relatively high titer of beta antibodies in the maternal serum. Here one may postulate that the beta antibodies were almost entirely of the bi valent variety.

#### STATISTICAL EVIDENCE CONCERNING THE ROLE OF THE A-B FACTORS IN ICTERUS PRECOX

One of the reasons why the role of isosensitization to the A and B agglutinogens in producing fetal and neonatal morbidity and mortality was not recognized earlier is that the manifestations usually are mild, and cases such as those described at the beginning of this article are rare. In typical cases, the baby develops only a mild jaundice, usually on the first or second day of life, and there may be a slight fall in the hemoglobin concentration. The disease is then arrested and recovery usually occurs without treatment in four to five days, although in more severe

TABLE 10—*Relative Frequency of Compatible and Incompatible Matings in Relation to the Clinical Manifestations in the Fetus and Newborn*

Clinical manifestation	Total number of cases	Compatible		Incompatible	
		Number	Per cent	Number	Per cent
Unexplained neonatal jaundice or anemia	94	19	20.2	75	79.8
Two or more abortions	89	43	47.3	46	52.7
Erythroblastosis due to Rh sensitization	282	232	82.2	50	17.8
Miscellaneous*	377	238	63.3	139	36.7
Theoretical distribution in random matings	—	—	65	—	35

\* These comprise all normal and abnormal infants not included in the other categories.

cases one or more blood transfusions may be required. This syndrome has been designated *icterus precox* by Halbrecht,<sup>8</sup> in order to distinguish it from so-called physiologic icterus. Among 60 cases of this type, Halbrecht found that in 57 (95 per cent) the blood group of the infant was incompatible with that of the mother, in contrast with the frequency of only 26.5 per cent incompatible in infants among 2,000 normal maternity cases. This finding has been confirmed by Wiener et al.<sup>12</sup> who found that 34 (or 81 per cent) of 42 infants with mild jaundice and anemia belonged to blood groups incompatible with those of their mothers.

When the blood of the child is not available for testing, statistical data concerning the role of the A and B factors may be gathered by comparing the blood groups of the father and the mother. If the father's blood group is incompatible with that of the mother, the mating is said to be incompatible; if the father's blood group is compatible, the mating is said to be compatible. In table 10, we have indicated the relative incidence of incompatible and compatible matings in a variety of clinical conditions including *icterus precox*, typical erythroblastosis, and repeated abortions. Among normal pregnancies the expected incidence of incompatible matings is about 35 per cent. In contrast to this, among 94 cases of

unexplained neonatal jaundice and anemia, as many as 79.8 per cent of the matings were incompatible, indicating that A-B sensitization must have played a part in at least a majority of these cases. On the other hand, among 282 families with erythroblastotic infants due to Rh sensitization only 17.8 per cent of the matings were incompatible, indicating that group incompatibility of the infant's blood reduces the likelihood of sensitization to the Rh factor.\* Incidentally, in patients with two or more unexplained early abortions the incidence of incompatible matings is 52.7 per cent, which is higher than the frequency of incompatible matings in the normal population, and suggests that a certain percentage of these may perhaps be due to A-B sensitization as has been previously pointed out by Levine<sup>35, 44</sup>

#### THE SECRETOR TYPE IN RELATION TO A-B SENSITIZATION

In order further to test Levine's theory that A-B sensitization occurs only in babies of the nonsecretor type, the saliva specimens were examined in a series of 14 families with typical serologic and clinical findings of erythroblastosis due to A or B sensitization. The findings on these families are listed in table 11. It will be seen that all of the affected babies in these families were secretors, which would seem to disprove Levine's contention. Of particular interest are families 6 and 7 in which the babies are secretors even though the fathers are nonsecretors.

Our results suggest that it is actually a disadvantage for the baby to be a secretor since this increases the likelihood of sensitizing the mother (cf. Smith<sup>45</sup>). Presumably the group substances in solution in secretions could traverse the placental barrier more readily than intact red cells. Moreover, smaller amounts of solutions containing group substances are sufficient to sensitize than are intact red cells. For example, as little as 0.2 cc. of autoclaved saliva administered intramuscularly can stimulate a rise in isoagglutinin titer.<sup>46</sup>

Parenthetically, the cases listed in table 11 show a remarkably high incidence of cerebral sequelae. It will be important to ascertain if this is maintained in a larger series of cases of A-B sensitization.

#### COMMENT

Summarizing the evidence which has been presented concerning the pathogenesis of erythroblastosis due to A-B sensitization, the first requisite is that the maternal serum contain univalent alpha or beta antibodies. A high percentage of normal individuals possess such univalent alpha and beta antibodies, but they are usually of only moderate or low titers. In cases of Rh sensitization, even a weak univalent Rh antibody is often sufficient to cause disease in an Rh-positive fetus, though the severity is correlated with the height of the antibody titer. On the other hand, that a low or moderate titer of univalent alpha or beta antibodies is usually relatively harmless to the infant suggests the presence of a special protective mechanism

\* It was previously suggested by one of us<sup>42</sup> that this phenomenon could be explained on the basis of competition of antigens. Another plausible explanation is that any group incompatible fetal blood which might leak into the maternal circulation would be rapidly eliminated before Rh sensitization could take place.<sup>43</sup>



TABLE 11—Incidence of the Secretor Types in Families with Erythroblastosis or Latens Præfix du AB Sensitization

Case No	Father	Mother	Pregnancies
1*	BMRh <sub>0</sub> Secretor	OMNRh (No anti Rh in serum)	1 BMNRh <sub>0</sub> ♀ secretor, normal. 2. BMNRh <sub>0</sub> ♂, secretor, erythroblastosis, treated by exchange transfusion.
2	A <sub>1</sub> MNRh <sub>1</sub> rh Secretor	OMNRh <sub>1</sub> Rh <sub>2</sub>	1 OMNRh <sub>1</sub> rh♀, normal 2. A <sub>1</sub> MNRh <sub>1</sub> rh♀, secretor jaundice, treated with transfusion.
3	A <sub>1</sub> MNRh Secretor	OMNRh <sub>0</sub>	1 AMNRh <sub>0</sub> ♂ secretor jaundice 2nd day moderate anemia, recovered without treatment
4	BMRh <sub>1</sub> Secretor	OMNRh <sub>1</sub>	1 Premature twins both♀ one stillborn. Other twin, BMRh <sub>1</sub> rh secretor, jaundice and anemia, transfused 4 times microcephalic and amaurotic, idiotic, died at 5 years
5	BMNRh Secretor	OMNRh <sub>1</sub> Rh <sub>1</sub>	1 BMRh <sub>1</sub> rh♂ secretor, normal 2. Macerated stillborn ♂ 3 BMNRh <sub>1</sub> rh♂ secretor jaundice, mild anemia transfused once, recovered.
6	A <sub>1</sub> MRh <sub>1</sub> Rh <sub>1</sub> Non secretor	OMNRh <sub>1</sub> rh	1 A <sub>1</sub> MNRh <sub>1</sub> rh♂ secretor, normal 2. A <sub>1</sub> MNRh <sub>1</sub> rh♂ secretor jaundice and anemia transfused 4 times, recovered completely
7	A <sub>1</sub> BMNRh <sub>1</sub> rh Nonsecretor	OMNRh <sub>1</sub> rh	1 A <sub>1</sub> MRh <sub>1</sub> rh♀ normal 2. AMRh <sub>1</sub> rh♀, secretor slight jaundice, recovered spontaneously
8	BNrh Secretor	OMNRh <sub>1</sub> rh	1 BMNRh♂ secretor normal 2. BMNRh <sub>1</sub> rh♂ secretor jaundice anemia, transfused recovered
9	A <sub>1</sub> MNRh <sub>1</sub> Rh <sub>2</sub> Secretor	OMRh <sub>2</sub> rh	1 OMNRh <sub>2</sub> Rh <sub>2</sub> ♂, normal. 2. OMRh <sub>1</sub> rh♀ normal 3 A <sub>1</sub> MNRh <sub>1</sub> rh♀ secretor jaundiced 2nd day transfused 2 times subsequently exhibited mental retardation
10	A <sub>1</sub> MNRh Secretor	OMNRh <sub>2</sub> rh	1 A <sub>1</sub> MRh <sub>2</sub> ♂ secretor jaundice, transfused once recovered completely 2. Jaundiced died on the 5th day
11	A <sub>1</sub> MRh <sub>1</sub> Rh <sub>1</sub> Secretor	OMNRh <sub>1</sub> rh	1 A <sub>1</sub> MRh <sub>1</sub> rh♀ secretor anemia and mild jaundice transfused 3 times, complete recovery
12†	A <sub>1</sub> Mrh Secretor	ONRh <sub>1</sub> Rh <sub>1</sub> (No anti hr' in serum)	1 A <sub>1</sub> MNRh <sub>1</sub> rh♀, secretor, normal. 2. OMNRh <sub>1</sub> rh♂, normal 3 A <sub>1</sub> MNRh <sub>1</sub> rh♀, secretor normal 4. A <sub>1</sub> MNRh <sub>1</sub> rh♂ secretor jaundiced 2nd day Icterus index = 160 units recovered. 5 A <sub>1</sub> MNRh <sub>1</sub> rh♀ secretor jaundice and anemia, treated by 2 exchange transfusions recovered
13	A <sub>2</sub> BMNRh <sub>2</sub> Non secretor	OMNRh <sub>2</sub> rh	1 BMNRh <sub>2</sub> ♂ secretor normal 2. BMNRh <sub>2</sub> ♂, secretor, jaundice, transfusion recovered
14	A <sub>1</sub> BMRh <sub>1</sub> rh Secretor	OMNRh <sub>1</sub> rh	1 A <sub>1</sub> MRh <sub>1</sub> rh♂ secretor neonatal jaundice lasting 3 days recovered completely 2. A <sub>1</sub> Mrh♀, secretor jaundice and anemia transfused once subsequently exhibited mental and physical retardation.

\* Same as case 1 reported in detail at beginning of paper

† For clinical details concerning this case we are indebted to Dr Irving L. Samuels of the Grasslands Hospital Valhalla N. Y.

This appears to consist primarily in the incomplete state of development of the A and B agglutinogens in the red cells of the newborn infant or fetus. In contrast, the Rh-Hr agglutinogens are fully developed at birth.

Some recent unpublished observations suggest a second consideration which may possibly contribute to the difference in behavior of cases of erythroblastosis due to A and B sensitization as compared with those caused by Rh sensitization. Specific alpha and beta antibodies may be classified in two different categories, namely, homospecific and heterospecific. Homospecific antibodies can be defined as those produced by injecting animals or human beings with the identical antigen subsequently to be used in the *in vitro* tests. Injections of a foreign antigen sometimes stimulate heterospecific antibodies which cross react because of the structural similarity of the two different antigens. For example, homospecific anti-A serum can be produced by immunizing human group B or group O individuals with group A blood, while heterospecific anti-A serum can be produced by injecting rabbits with sheep blood which contains an antigen chemically related to human A substance. The homospecific antibody presumably fits the corresponding antigen precisely, in the manner that a key fits its corresponding lock, while a heterospecific antibody can be conceived as fitting the antigen like a skeleton key would fit a number of locks of related structure. It seems probable that the combination of a homospecific antibody with its corresponding antigen would be much more avid than that of a heterospecific antibody, so that the latter could possibly be more readily eluted or washed off.

It is characteristic of icterus precox that the firstborn is often affected, and this fits well with the observations presented in this paper demonstrating that univalent alpha and beta antibodies occur in the majority of normal individuals. As has already been pointed out, these are presumably of heterospecific origin, which might partially account for the benign nature of the syndrome. On the other hand, the Rh antibodies responsible for typical erythroblastosis are invariably of homospecific origin, which, in accordance with this hypothesis, would explain the severity of the manifestations. Continuing in the same vein it would be expected that when the alpha and beta antibodies are homospecific, they could also produce severe erythroblastosis. Thus, in our Case 1 treated by exchange transfusion, the first group B baby was normal while the second group B baby was severely affected, presumably due to the active immunization of the mother to the B agglutinogen of the first baby. The second case is even more interesting because the first group A baby was only moderately affected (icterus precox) due to preformed univalent heterospecific alpha antibodies in the maternal serum, while the second baby was severely affected due to active sensitization of the mother resulting after the birth of the first group A baby, with the formation of homospecific alpha antibodies. Further work is in progress to test the validity of concepts.

All of the affected infants tested proved to be secretors, disproving the hypothesis that the disease occurs only in nonsecretors. In fact, the secretor type appears to predispose somewhat to the occurrence of the disease since it increases the chance of sensitization of the mother. A far smaller volume of group substance in solution is required to sensitize than is whole blood. It seems significant that in monkeys

the A and B agglutinogens are lacking from the red cells, but four groups do occur based on the presence of group substances in secretions.<sup>47-48</sup> The evolutionary passage of A and B substances from secretions to the red cells in higher primates may thus be considered as an unfavorable step, since it predisposes to fetal and neonatal death due to A and B sensitization. The mutation which produced the nonsecretor type, presumably at a later date, may be considered as a favorable one, since it reduces the chances of sensitizing the mother. In this connection, it seems significant that the highest incidence of the nonsecretor type is found in negroes,<sup>49</sup> who in many respects are the most highly differentiated members of the human race.

The use of soluble A and B substances for the treatment of A-B sensitization in babies had previously been suggested.<sup>49</sup> However, the results of treatment with these substances in the two cases presented here were disappointing. Actually, this should not be completely unexpected, because before the affected infant is born the antibodies have already been bound by the red cells from which they would be displaced only with difficulty. Our findings do indicate the possibility of anticipating the disease by antenatal tests done on the mother's serum. However, the incidence of typical erythroblastosis due to A-B sensitization is very low, and in most cases the manifestations in the child are mild, so that it would be hardly worth while to include alpha and beta titrations as well as anti-Rh as a routine in all antenatal cases at the present time.

#### SUMMARY

Two unusual cases of severe erythroblastosis due to A and B sensitization have been presented. When injections of A and B group substances failed to arrest the disease, exchange transfusions were carried out, using 900 to 1,000 cc. of fresh group O blood. In each case the response was prompt and dramatic, although the convalescence in one was prolonged by an intercurrent diarrhea. Both infants have made complete recoveries and have developed normally both physically and mentally.

Observations have been presented regarding the pathogenesis of erythroblastosis and icterus precox due to A-B sensitization. The following conclusions seem to be warranted on the basis of the evidence presented:

1. The greatest majority of cases of jaundice and anemia of the newborn that cannot be explained on the basis of Rh incompatibility are caused by incompatibility of the major blood groups.
2. High maternal alpha and beta antibody titers *per se* are not necessarily correlated with disease in the infant.
3. Univalent alpha and beta antibodies present in the maternal serum traverse the placenta and are the cause of the disease in the infant. Bivalent antibodies are held back by the intact placenta and play no or hardly any role in the causation of the disease. Univalent alpha and beta antibodies are demonstrable in the sera of a large proportion of normal individuals.
4. A-B sensitization in pregnancy occurs mainly when the infant belongs to the secretor type.
5. A theory is suggested that the quality of the alpha and beta antibodies

namely, whether they are homospecific or heterospecific, may affect the severity of the manifestations in the infant

Technics of titrating alpha and beta and Rh antibodies are described and discussed. A table has been prepared which converts antibody titers into concentrations of immune globulin in the serum, and demonstrates the impossibility of certain extravagantly high titers claimed in the literature

#### REFERENCES

- <sup>1</sup> WIENER, A S, WEXLER, I B AND GAMBIN, E Hemolytic disease of the fetus and newborn infant with special reference to transfusion therapy and the use of the biological test for detecting Rh sensitization *Am J Dis Child* 68 317-323 1944
- <sup>2</sup> — AND — The use of heparin when performing exchange transfusions on newborn infants *J Lab & Clin Med* 31 1016-1019 1946
- <sup>3</sup> —, —, AND GRUNDFAST, T H Therapy of erythroblastosis fetalis with exchange transfusion *Bull New York Acad Med* 23 207-220 1947
- <sup>4</sup> — — AND SHULMAN, A Therapy of severe erythroblastosis fetalis with repeated and massive exchange transfusions *Am J Clin Path* 18 141-151, 1948
- <sup>5</sup> — AND — Results of therapy of erythroblastosis with exchange transfusion *Blood* 4 1-35, 1949.
- <sup>6</sup> POLATES, S H Erythroblastosis fetalis unrelated to the Rh factor. Isoimmunization of group O mothers by group A children Meeting N Y Path Soc, Jan, 1942, Nov 29 1945
- <sup>7</sup> — Erythroblastosis in mothers with Rh positive blood *Am J Dis Child* 69 99-102, 1945
- <sup>8</sup> HALBRECHT, I Role of hemagglutinins anti A and anti B in the pathogenesis of jaundice of the newborn *Icterus neonatorum precox* *Am J Dis Child* 69 208-209 1944
- <sup>9</sup> WIENER, A S Recent advances in knowledge of the Rh blood factors, with special reference to the clinical applications *Tr and Studies Coll Phys Phil* 13 105-122, 1945
- <sup>10</sup> POLATES, S H Erythroblastosis fetalis unrelated to the Rh factor *Am J Clin Path* 15 467-470 1945
- <sup>11</sup> — AND McNALLY, J, JR Iso-immunization with the A and B factors and its relation to hemolytic disease of the newborn *Am J Clin Path* 18 375-386 1948
- <sup>12</sup> WIENER, A S SONN, E B, AND HURST, J G Pathogenesis of erythroblastosis fetalis III Illustrative case histories of A B sensitization Studies on individual differences in human blood and their practical applications, Paper No 1 Brooklyn N Y, July 15 1946
- <sup>13</sup> —, AND — Permeability of the human placenta to iso-antibodies *J Lab & Clin Med* 31 1022-1024, 1946
- <sup>14</sup> GRUNDBACH, A AND GASSER, C A B-O Inkompatibilitäten und Morbus haemolyticus neonatorum *Helv Paed Acta* 3 447-474, 1948
- <sup>15</sup> TORTORA, M Icterus praecox da sensibilizzazione materna verso il fattore A. *Arch di Ost e Gin* 52 436-438, 1948
- <sup>16</sup> WIENER, A S Recent developments in the knowledge of the Rh Hr blood types Tests for Rh sensitization *Am J Clin Path* 16 477-497 1946
- <sup>17</sup> BELKIN, R B AND WIENER, A S Demonstration of the properties A B M, N and Rh in red cell stroma *Proc. Soc Exptl Biol & Med* 56 214-217 1944
- <sup>18</sup> WIENER, A S AND SONN-GORDON, E B Simple method of preparing anti Rh serum in normal male donors *Am J Clin Path* 17 67-70 1947
- <sup>19</sup> HILL, J M HABERMAN, S AND JONES, F Hemolytic Rh immune globulins Evidence for a possible third order of antibodies incapable of agglutination or blocking *Blood* (Special issue No 2) 80-100 (Jan) 1948
- <sup>20</sup> WIENER, A S The Rh factor in immunological reactions *Ann Allergy* 6 293-305 1948
- <sup>21</sup> — AND HANDMAN, L Simple method of detecting univalent antibodies in sera containing potent bivalent antibodies, with remarks concerning the pathogenesis of erythroblastosis fetalis *Exper Med & Surg* 6 416-427 1948
- <sup>22</sup> — A new test (blocking test) for Rh sensitization *Proc Soc Exptl Biol & Med* 56 173-176 1944
- <sup>23</sup> — AND HURST, J G A new sensitive test (direct test) for Rh blocking antibodies *Exper Med & Surg* 5 285-295 1948

- <sup>24</sup> COOMBS R R A MOURANT A E AND RACE R R A new test for the detection of weak and incomplete Rh agglutinins *Brit J Exp Path* 26 255-260 1945
- <sup>25</sup> MORRESCHI, C Neue Tatsachen über die Blutkörperchenagglutination *Centralbl f Bakteriol* 46 49-51 1908
- <sup>26</sup> ROSENFELD R VOGEL, P AND WIENER, A S Unpublished observations
- <sup>27</sup> WITERSKY E MOHN J F HOWLES D J AND WARD H M A simple method for the concentration of Rh agglutinins *Proc Soc Exper Biol & Med* 61 1-4 1946
- <sup>28</sup> WEISL, B A G Possible hemolytic anemia of the newborn due to high titer anti A agglutinin in a group O mother *Am J Obst & Gynec* 56 1002-1004 1948
- <sup>29</sup> BARRETT C D JR AND TRIPP J A A method of adjusting the antigen antibody ratio prior to the determination of pneumococcal antibody nitrogen *J Immunol* 43 311 1942.
- <sup>30</sup> KABAT E A AND BREZER A E Immunochemical studies on blood groups I Estimation of A and B isoantibodies in human serum by the quantitative precipitation method *J Exp Med* 82 207-215 1945
- <sup>31</sup> WIENER A S HURST J G AND HANDMAN L Emploi de gélatine et d'autres produits de remplacement pour la titrage des anticorps Rh univalent par la réaction de coagulation *Rev de Hématol* 3 3-12, 1948
- <sup>32</sup> POLAYES S H, LUBIN, S, AND McNALLY J Antenatal blood group determination *Am J Obst & Gynec* 55 524-526, 1948
- <sup>33</sup> WIENER A S HURST J G AND SONN E. B Studies on the coagulation reaction with special reference to the nature of coaglutinin *J Exp Med* 86 267-284 1947
- <sup>34</sup> KEMP T Recherches sur le degré de sensibilité des nouveau nés (sang du cordon ombilical) vis-à-vis des isohémagglutines du sang des adultes *Acta Pathol et Microbiol Scand* 7 146-160 1930.
- <sup>35</sup> TOVEY G H A study of the protective factors in heterospecific group pregnancy and their role in the prevention of hemolytic disease of the newborn *Jour Path & Bact (London)* 57 295-304, 1945
- <sup>36</sup> LEVINE, P Genetic and constitutional causes of fetal and neonatal morbidity *Ann New York Acad Sc* 46 939-961 1946
- <sup>37</sup> DOCTER J M Kernicterus Neurological sequelae of erythroblastosis fetalis *J Pediat* 27 327-334 1945
- <sup>38</sup> WIENER A S Blood Groups and Transfusion ed 3 Springfield Ill C. C. Thomas 1943
- <sup>39</sup> RENKONEN K O Studies on hemagglutinins present in seeds of some representatives of the family of leguminosae *Ann Med Exp et Biol Fenn* 26 66-72, 1948
- <sup>40</sup> POLAYES S H LEDERER M. AND WIENER A S Studies in isohemagglutination II The Landsteiner blood groups in mothers and infants *J Immunol* 47 545-554 1929
- <sup>41</sup> WIENER A S AND SILVERMAN I J Permeability of the human placenta to antibodies A quantitative study *J Exp Med* 71 21-27 1940
- <sup>42</sup> YCAS M. Y WARREN Studies on the development of a normal antibody and of cellular antigens in the blood of sheep *J Immunol* 61 327-347 1949
- <sup>43</sup> WIENER A S Competition of antigens in iso-immunization by pregnancy *Proc. Soc. Exper Biol & Med* 58 133-135 1945
- <sup>44</sup> MOLLISON P L. MOURANT A E AND RACE, R R The Rh blood groups and their clinical effects Medical Research Council Memorandum No 19 London 1948 P 21
- <sup>45</sup> LEVINE, P Serological factors as possible causes of spontaneous abortions The Abortion Problem Chapter VI Baltimore Md Williams & Wilkins 1944
- <sup>46</sup> SMITH G H Iso-agglutinin titers in heterospecific pregnancy *J Path & Bact* 57 113-122, 1945
- <sup>47</sup> WIENER A S SOBLE R AND POLIVKA H Simple method of preparing potent blood grouping sera *Proc Soc. Exper Biol & Med* 58 310-312, 1945
- <sup>48</sup> CANDELA P B AND WIENER A S AND GOSS, L. J New observations on the blood group factors in Simiidi and Cercopithecidae *Zoologica* 25 513-521 1940
- <sup>49</sup> WIENER A S CANDELA P B AND GOSS L. J Blood group factors in the blood organs and secretions of primates *J Immunol* 45 229-235 1942.
- <sup>50</sup> — AND BRODY M. The encephalopathy (kernicterus) of erythroblastosis fetalis Its serological diagnosis and pathogenesis *Am J Ment Deficiency* 51 1-14 1946

# THE EFFECT OF STASIS OF BLOOD IN VARICOSE VEINS ON ERYTHROCYTE FRAGILITY, WITH ACCOMPANYING STUDIES COMPARING RED CELLS AND OTHER BLOOD ELEMENTS WITH CUBITAL VEIN BLOOD

By STACY R. METTIER, M.D., JOHN C. WEAVER, M.D., AND  
ALICE F. MCBRIDE, B.A.

IT HAS been demonstrated by Fahræus<sup>1-4</sup> that normal red blood cells have a tendency to become spheroidal in shape after standing in vitro at body temperature. Gänsslen,<sup>5</sup> Haden<sup>6</sup> and Castle and Daland<sup>7</sup> have shown that spheroidal red cells are more susceptible to osmotic hemolysis than are normal corpuscles. Haden found that normal red cells when suspended in graded hypotonic salt solutions become progressively more globular as the solution becomes more hypotonic, and that there is a direct relationship between the volume thickness index and fragility of the red blood cells. Ham and Castle<sup>8</sup> and Tsai, Lee and Wu<sup>9</sup> have further stated that red cell fragility is increased by stasis of the blood in vitro at body temperature. These investigators found that between one-half and two and one-half hours of stasis was necessary before the first manifestation of increased fragility appeared. Spontaneous hemolysis appeared after approximately twelve hours of stasis.

During stasis of blood at body temperature there is also an increase in packed cell volume due to the development of spheroidal cells.<sup>8, 10-11</sup> There is also evidence that under some conditions, in vivo stasis produces increased red cell fragility. Waller<sup>12</sup> and Cermick<sup>13</sup> found an increase in red cell fragility in capillary blood following tourniquet stasis, but Waller found no increased fragility in blood removed from the cubital vein under conditions of stasis except after expressing capillary blood into the veins.

There is evidence that concentration and stasis of red blood cells occur normally in the spleen.<sup>14-16</sup> Red cells obtained from the splenic vein were found to be more fragile when suspended in hypotonic salt solutions than red cells from blood in other veins.<sup>8, 9</sup> As further proof that in vivo stasis may cause red cells to become more fragile, Tsai and co-workers<sup>9</sup> found that osmotic fragility of red cells removed from both the splenic and renal veins increased progressively following stasis produced by occlusion of the veins and arteries of the spleen and kidney.

Ham and Castle<sup>8, 10, 17-18</sup> have attached great importance to the effects of stasis on red cells and consider this factor to be the common denominator in many of the anemias due to hemolysis. They believe that erythrosthesis in the spleen is probably the mechanism producing increased blood destruction in the hemolytic anemias with increased red cell fragility, and also, that an unusual degree of erythrosthesis might account for some hemolytic anemias in which there is normal or secondarily increased red cell fragility. A number of investigators<sup>9, 19-21</sup> have shown that the red cells usually become less fragile to hypotonic salt solutions

following splenectomy Ham and Castle interpret the beneficial effect of splenectomy as being due to the removal of the organ in which a large degree of red cell stasis (and thus increased fragility) occurs. They explain certain anemias associated with splenomegaly on the basis of a probable increase in normal splenic function with respect to erythrostatics.

In an attempt to evaluate further the effect of *in vivo* stasis as a possible mechanism for increasing red cell fragility, the present investigation was undertaken to measure the osmotic fragility of red blood cells from veins in 20 patients without any known hemolytic tendency or other blood dyscrasia. Although the degree of stasis in varicose veins is not known it seems well established that the movement of blood in varicosities is very sluggish. Ochsner and Mahorner<sup>5</sup> visualize the leg with varicose veins as having a circulation of its own. They consider the possibility of a given blood cell remaining in the venous system of the leg indefinitely, coming up each time perhaps to the opening of the saphenous where it again becomes one of the unhappy ones to fall through the opened sluices peripheral in the superficial venous system.

McPheeters and Rice<sup>26</sup> studied the direction of blood flow in leg varicosities and discussed in detail the movement of lipiodol injected into the varicosities of two patients with a positive Trendelenburg test. One subject was recumbent and the other sitting with legs horizontal. In both cases the injected lipiodol remained stationary until the patient tensed the abdominal muscles or moved the feet, following which the lipiodol was seen to move distally. In their experience the dye never moved centrally. However, Schmier<sup>27</sup> and Heller<sup>28</sup> observed that injected radio opaque material moved in a central direction. Heller determined the specific gravity of blood removed from the varicose vein under observation and then injected radio opaque dye of the same specific gravity. He found in patients having varicose veins and competent valves that the circulation was directed centrally but at a slower rate than in the normal control. In patients with incompetent valves the flow was nearly stationary but after the patient remained standing for some time a very slow upward flow developed. Coughing or straining rapidly reversed the flow and forced the opaque substance distally. He also noted that when the patient first stands after being in a supine position there is a surge of blood down the varicose vein. While there is no definite proof that a significant quantity of blood stagnates in the varicose vein for hours it is evident that abnormal erythrostatics does occur.

### METHOD

The patients used in this study had all been previously examined in the Varicose Vein Clinic of the University of California Medical Center. Each patient was requested to stand quietly for a period of at least fifteen minutes. Blood was then drawn from a tortuous dilated superficial vein, usually on the calf, and immediately afterward a similar sample was obtained from the cubital vein without the aid of a tourniquet.

The following studies were made on the two specimens of blood: (1) The osmotic fragility of the red cells. (2) The packed cell volume. (3) Hemoglobin, red blood count, white blood count and platelet count (Rees and Ecker method<sup>29</sup>). (4) Plasma protein (Falling drop method).

All of the laboratory determinations were performed by one of us.

The valves of the long saphenous veins were incompetent in each of the patients tested (Sp, Di, and Wh<sub>1</sub> were not tested). The clinical degree of tortuosity and dilatation of each patient is indicated in table 1. One patient (Di) had a varicose ulcer. There was neither evidence of congestive heart failure nor obvious blood dyscrasia in any of the patients studied.

## RESULTS

1 *Hypotonic fragility* The resistance of the red blood cells to hypotonic solutions of saline was determined on blood from the cubital and varicose veins of 19 otherwise healthy patients. The fragility of the red blood cells from the varicose veins was not significantly different from the fragility of red cells

TABLE 1

Name	Red Cell Count (Millions)		Packed Cell Volume		Total Protein		Red Cell Fragility		Degree of Viscosity
	Arm	Varicose	Arm	Varicose	Arm	Varicose	Arm	Varicose	
Re	4.07	4.20	44	42			48-36	44-32	++++
Fr	4.17	4.18	42	44	6.70	7.32	44-34	44-34	+++
McM	4.23	4.28	42	42	7.39	7.77	48-34	48-34	++++
St	4.03	4.33	40.5	40.5	6.87	6.87	50-38	50-36	+++
Ke	5.21	5.10	47	48	6.15	6.39	46-36	46-36	+++
Sp	4.33	4.36	47	48	6.66	6.56	46-32	47-32	+++
Va	4.72	5.43	45	44	6.22	5.73	46-34	44-30	++++
Di	4.28	4.63	46	46	6.49	6.42	42-30	42-32	+++
Se	4.40	4.34	44.5	46	6.15	6.08	44-32	42-32	++++
Whe	4.45	4.49	39.5	40	6.18	6.36	44-32	42-32	+++
Bro	4.08	4.23	43.5	43	5.94	5.87	42-32	42-30	++++
Ma	4.89	4.59	47	47	6.25	6.22	46-32	44-32	++
Le	4.37	4.52	44	44.5	6.32	6.52	48-36	48-38	++
Bri	4.23	4.39	41	42	6.18	6.49	42-32	44-32	+++
Os	4.60	4.53	45	44			46-36	44-34	+++
Win	4.75	4.88	47	47	5.94	6.01	48-38	48-38	+++
Mo	4.72	4.71	37	40.5	5.90	6.15	46-34	46-34	+++
Wh <sub>1</sub>	4.71	4.82	48	49			50-38	48-38	++++
Wil	4.27	4.55	41.5	43	5.46	5.63	44-34	44-34	+++
Jn	4.43	4.55	49	52	6.39	7.14	42-32	42-32	++
	88.94	91.11	880.5	892.5	107.19	109.53			

obtained from the cubital veins (table 1). In each patient the red blood cell fragility fell within the normal range in both the varicose vein and the cubital vein specimens.

2. *Red cell count, packed cell volume, serum protein, hemoglobin, white cell count and platelet count* The increased pressure in varicose veins should cause fluid transudation into the tissues which would be expected to produce hemoconcentration of the varicose vein blood. However, Erb and Tickense<sup>29</sup> found no increase in red cells, red cell fragility, white cells or platelets in blood from varicose veins as compared with cubital vein blood.

(a) Our results show a small but significant increase in red blood cell count in blood from varicose veins as compared with the cubital vein blood. Statistical calculations show  $P < 0.05$ ; the mean difference being 108.5 million red cells. This is indicative of a minor degree of hemoconcentration.

(b) Surprisingly enough the packed cell volume of varicose vein blood was only suggestively higher.

Statistical analyses of our data were made by Dr. John C. Talbot of the University of California Medical Center.



than cubital blood ( $P$  slightly  $< 0.05$ ) If as result of stasis some red cell swelling had occurred some increase in packed cell volume would result and added to a certain amount of hemoconcentration it would be expected that the packed cell volume would increase out of proportion to the red cell increase.

(c) There was a suggestive increase in total serum protein in varicose veins compared with cubital veins ( $P$  somewhat  $> 0.05$ ) A more accurate technic or a larger series would be necessary to establish the significance of this apparent increase

(d) There was no significant difference in hemoglobin platelets or white cells in the varicose and cubital vein samples

### COMMENT

As mentioned earlier, the preponderance of evidence indicates a slow, steady progression of blood centrally in varicose veins with refluxes of blood following straining and coughing and other activities which increase the intra abdominal pressure. It seems likely, then, that the slow movement of blood through varicose veins does not produce stagnation comparable to that which has been shown necessary to produce red cell swelling and increased fragility in the test tube. In an attempt to establish roughly the duration that a measurable quantity of blood remains in the varicose vein, we, in collaboration with Doctors J. Hopper, Jr., and C. J. Mudrick, performed the following experiment:

Evans blue dye (T1824) was injected into a varicose vein of a patient with marked varicosities. No dye appeared in the cubital vein blood until two minutes after the injection. The dye concentration gradually increased and finally leveled off fifteen minutes after the injection. Dye did not appear in the varicose veins of the opposite leg until four minutes following injection and failed to reach the dye concentration of the arm in a period of thirty minutes. It has been shown that dye injected in an arm vein of normal subjects appears in samples of blood taken from the other arm within thirty seconds and levels off within three to four minutes.<sup>21</sup> Obviously our experiment in one patient and without controls has no comparative value but it does indicate that in this patient a certain amount of stasis occurred in the varicosity for about fifteen minutes. Further experiments on this problem are in progress.

Our data permit no final conclusions regarding the importance of the factor of stasis in hemolytic diseases. However, the lack of increased red cell osmotic fragility under the conditions of stasis that occur in varicose veins suggests that erythrosthesis of a moderate degree does not play a major part in most hemolytic diseases. We are inclined to agree with the viewpoint of Dameshek and Miller<sup>22</sup> that the hemolytic states are due to a number of different causes such as hemolysins, agglutinins and inherited red cell abnormalities with such supplementary factors as stasis, trauma, and possibly, chemical and hormonal changes augmenting the occurrence of hemolysis. It seems likely that several factors are operating at once. For example, in the presence of hemolytic disease, increased stasis and increased trauma to the red cells might be expected to produce some increase in the degree of hemolysis. It also seems likely that in order for stasis appreciably to augment hemolysis in any given hemolytic syndrome it is necessary for stagnation to occur over a prolonged period of time. Tsai<sup>9</sup> showed that increased red blood cell fragility did not appear until stasis had been present for one-half hour to two and one-half hours and that hemolysis did not begin until about twelve hours of stasis. Also in

our experiment and in Waller's<sup>17</sup> a degree of stasis beyond that normally existing did not produce a significant increase in fragility in blood from veins. Therefore, the amount of stasis present in congestive failure or produced by increased blood viscosity caused by the increase in globulin in infections as suggested by Castle<sup>8, 17</sup> would hardly seem sufficient to produce hemolysis. It is probable that the spleen is the only organ in the body in which stasis, sufficient to cause a significant increase in hemolysis, might occur.

The absence of a greater degree of hemoconcentration than we found in varicose vein blood is difficult to understand. Beecher<sup>33</sup> found a gross filtration pressure of 50 cm. of water in excess of the colloid pressure of the blood in varicose veins and concluded that normal resorption of tissue fluid at the venous end of the capillary was impossible and all tissue fluid must be carried off by the lymphatics. This should result in marked hemoconcentration but our experiments showed evidence of only mild hemoconcentration. Obviously, factors are involved which have not been adequately explored.

Our results confirm the findings of Erb and Tiefensee<sup>30</sup> that there is no significant increase in white cells, platelets, and red cell fragility in blood from varicose veins as compared with cubital vein blood. However, our finding of a significant increase in red cells in the varicose vein is at variance with their conclusion that the red cells were not significantly higher than in the cubital vein.

#### SUMMARY AND CONCLUSIONS

1. Blood from varicose veins was compared with cubital vein blood in 20 patients in order to determine whether or not the degree of stasis present in varicose veins would increase red cell fragility. Corollary studies consisted of comparative determinations of red cells, hemoglobin, packed cell volume, white blood cells, platelets and serum proteins.

2. There was no increase in red cell fragility in the varicose vein specimen, indicating that the theory that minor degrees of intravascular erythrosthesis contribute substantially to some of the hemolytic anemias is untenable.

3. There was a small but statistically significant elevation in red cells per cu mm in varicose vein blood as compared with blood from cubital veins. There was a suggestive, but not significant, increase in packed cell volume and serum protein in the varicose vein samples. The evidence indicates a mild degree of hemoconcentration.

4. White cells, platelets and hemoglobin determinations were found to have the same values in varicose vein blood as in blood from the cubital vein.

#### REFERENCES

- <sup>1</sup> FAHRAEUS, R. The suspension stability of the blood. *Acta Med Scandinav* 55: 1, 19-1.
- <sup>2</sup> ———. The suspension stability of the blood. *Physiol Rev* 9: 41, 1929.
- <sup>3</sup> ———. The erythrocytes plasma interface. *Lancet* 630, 1939.
- <sup>4</sup> BERGENHEIM, B. AND FAHRAEUS, R. Ueber spontane Hamolysinebildung in Blut unter besonderer Berücksichtigung der Physiologie der Milz. *Ztschr f d ges exper Med* 97: 555, 1936.
- <sup>5</sup> GANSSLEN, M. Über hamolytischen Ikterus. *Deutsch Arch F Klin Med* 14: 210, 1922.

- <sup>6</sup> HADEN R L The mechanism of the increased fragility of the erythrocytes in congenital hemolytic jaundice *Am J M. Sc* 188 441, 1934
- <sup>7</sup> CASTLE W B, AND DALAND, G A Susceptibility of mammalian erythrocytes to hemolysis with hypotonic solutions A function of differences between discoidal volume and volume of a sphere of equal surface *Arch Int Med* 69 949 1937
- <sup>8</sup> HAM T H, AND CASTLE, W B Studies on destruction of red blood cells Relation of increased hypotonic fragility and of erythrostatics to the mechanism of hemolysis in certain anemias. *Proc. Am Philosoph Soc.* 82. 411 1940
- <sup>9</sup> TSAI C, LEE J S, AND WU C H The role of splenic action in altering erythrocyte fragility *Chinese J Physiol* 15 165 1940
- <sup>10</sup> HAM T H, AND CASTLE W B Mechanism of hemolysis in certain anemias Significance of increased hypotonic fragility and of erythrostatics (Abstract) *J Clin Investigation* 19 788 1940
- <sup>11</sup> TSAI, C., CHEN, C J AND CHIN K Y Observation on the various factors influencing the increase of erythrocytic fragility induced by stasis *Am J Physiol* 138 519 1943
- <sup>12</sup> WALLER J Cause of increased fragility of erythrocytes in congestive heart failure *Proc. Soc. Exper Biol & Med* 42 64, 1939
- <sup>13</sup> CORMICK, J A study of the quantitative fragility test in children *Arch Dis Child* 17 227 1941.
- <sup>14</sup> BARCROFT, J AND BARCROFT, H Observations on the taking up of carbon monoxide by the hemoglobin in the spleen *J Physiol* 58 138 1923
- <sup>15</sup> HANAK, A. AND HARKAVY J Observations on the taking up of carbon monoxide by the hemoglobin of the spleen *J Physiol* 59 121, 1924
- <sup>16</sup> KNISELY, M. H Spleen studies I Microscopic observations of the circulatory system of living unstimulated mammalian spleens *Anat. Rec* 65 23, 1936
- <sup>17</sup> HAM T H., AND CASTLE, W B Relation of increased hypotonic fragility and of erythrostatics to the mechanism of hemolysis in certain anemias *Tr A Am Physicians* 55 127 1940
- <sup>18</sup> — and — Studies on destruction of red blood cells Relation of intravascular stasis and of abnormal fragility of erythrocytes to the mechanism of hemolysis in certain hemolytic anemias. (Abstract) *Yearbook Philadelphia, Am Philosoph Soc*, 1939 Pp 228-229
- <sup>19</sup> PEARCE, R M., KRUMBHAAR, E B AND FRAZIER, C. H The Spleen and Anemia Philadelphia J B Lippincott Co 1918 Pp 42
- <sup>20</sup> ORAHOVAT D The spleen and the resistance of red cells *J Physiol* 61 436 1926
- <sup>21</sup> FRENCKELL, G AND NEKLUDOW, V N *Pflügers Arch* 220 356 1928 (cited by Tsai Lee and Wu 1940)
- <sup>22</sup> GORDON, A S, AND KLEINBERG W A study of the relation of the spleen to erythropoiesis and red cell destruction in the guinea pig *Am J Physiol* 118 757, 1937
- <sup>23</sup> SINGER K MILLER, E. B AND DAMESHEK, W Hematologic changes following splenectomy in man with particular reference to target cells hemolytic index and lysolecithin *Am J M. Sc.* 192. 171 1941
- <sup>24</sup> BOTTAZZI F La milza come organo emocatonistico *Sperimentale scg Biol* 48 433 1894
- <sup>25</sup> OCHSNER, A AND MAHORNBER, H Varicose Veins St Louis, C. V Mosby Co, 1939 Pp 57
- <sup>26</sup> MCPHERTERS H O, AND RICE C O Varicose veins The circulation and direction of the venous flow *Surg Gynec & Obst* 49 29 1929
- <sup>27</sup> SCHMIDT A A Sclerolyzing injections in varices of the lower extremities *J.A.M.A.* 94. 1222, 1930.
- <sup>28</sup> HILLER R E The circulation in normal and varicose veins *Surg, Gynec. & Obst.* 74. 1118, 1942.
- <sup>29</sup> REES H M AND ECKER E E An improved method for counting blood platelets *J.A.M.A* 80 621 1923
- <sup>30</sup> ERB K. H AND TIRFENSER K Untersuchungen über das Krampfaderblut *Beitr z. klin Chir* 152. 400 1931
- <sup>31</sup> BROWN, E HOPPER J JR MUDRICK C U AND SAMPSON J J To be published
- <sup>32</sup> DAMESHEK, W AND MILLER E B Pathogenic mechanisms in hemolytic anemias *Arch Int Med* 72 1 1943
- <sup>33</sup> BECHER H K. Adjustment of the flow of tissue fluid in the presence of localized sustained high venous pressure as found with varices of the great saphenous system during walking *J Clin Investigation* 16 733 1937

# THE ROLE OF STAPHYLOCOAGULASE IN BLOOD COAGULATION

## I THE REACTION OF STAPHYLOCOAGULASE WITH COAGULASE-GLOBULIN (CG) TO FORM COAGULASE-THROMBIN (CT)

By JOHN B. MIALE, M.D.

THE ABILITY of some strains of *Staphylococcus aureus* to clot oxalated plasma has long been recognized. This phenomenon was first reported by Loch<sup>1</sup> in 1903. Later, Gratia<sup>2-6</sup> concluded that a substance which he called 'staphylocoagulase' was produced by actively growing organisms and that this was the agent responsible for the coagulation of the plasma.

Attempts to define the nature and mode of action of staphylocoagulase have resulted in a great deal of conflicting data. Contradictory findings on the filtrability of this substance through bacterial filters have been reported by Gross,<sup>7-9</sup> Genou,<sup>10</sup> Vanbreuseghem<sup>11</sup> and Walston.<sup>12</sup> Lominski<sup>13</sup> found that he was unable to separate staphylocoagulase from the bacterial cells by filtration through Seitz and Chamberland filters, centrifugation, or by killing the organisms by heat and chloroform and ether vapor. He concluded that staphylocoagulase was formed only in the presence of living organisms and plasma. He therefore prepared what he called staphylocoagulase by adding plasma to the broth culture and filtering through a Chamberland L<sub>4</sub> candle.

The ability of staphylocoagulase to clot purified fibrinogen has also been debated, Much<sup>14</sup> claiming that it did not, while Gratia,<sup>5</sup> Cruickshank<sup>15</sup> and Walston<sup>12</sup> claimed that it did. Some of these conflicting opinions are probably due to the use of impure preparations of fibrinogen.

Smith and Hale<sup>16</sup> were the first to demonstrate the nature of the reaction. They showed clearly that staphylocoagulase could be obtained as a sterile cell-free culture filtrate, and that this was unable to clot fibrinogen unless an accessory factor present in tissue extracts and plasma was added. They termed this accessory factor an 'activator' of staphylocoagulase, and this concept is retained by Ferguson.<sup>19</sup> Lominski and Roberts<sup>17</sup> added the finding of a serum inhibitor, but it must be pointed out that the material which they called staphylocoagulase was not the native staphylococcal product, as is obvious from Smith and Hale's studies and from the data below. As this paper was being prepared, Kaplan and Spink<sup>18</sup> published their studies with living cultures of *Staphylococci* and confirmed and extended the above concepts.

This series of studies is an attempt to define the relationship of this bacterial substance to the mechanism of blood coagulation. The present paper presents data on the nature of the substance elaborated by staphylococci (*staphylocoagulase*) and observations on the nature of plasma factor with which it reacts (*coagulase*).

From the Laboratories of the Marshfield Clinic and St. Joseph's Hospital, Marshfield, Wisconsin.  
This study was aided during 1944-1946 by grants from the University of North Carolina—Watts Hospital Research Fund and since then by the Marshfield Clinic Research Foundation.

globulin, (CG) to form a thrombin-like substance which is referred to as *coagulation-thrombin*, (CI)

## EXPERIMENTAL

### I. Preparation of Staphylocoagulase

For critical experiments it is necessary to work with a sterile cell-free preparation of a known staphylocoagulase titer. Although it is possible to obtain material of higher titer in non-cell free preparations it is best to use staphylocoagulase prepared by the following method.

A suitable strain of *Staphylococcus aureus* is inoculated into 10 cc of Tryptose broth (Difco) and incubated at 37 C for six hours. The entire 10 cc are then inoculated into 500 cc of Tryptose broth, pH 7.4, and incubated at 37 C. for twenty-four hours. During this period, the pH of the culture is checked repeatedly (Beckman pH meter) and the pH maintained as near as possible at 7.0-7.4. After incubation, the culture is then filtered through a Berkefeld V candle and the filtrate (sterility checked) is distributed into smaller air-tight sterile containers and stored in the refrigerator (0-4 C). Very little loss of titer takes place over a period of several months. The titer of staphylocoagulase is determined by the serial dilution method previously described.<sup>20</sup> The quantitative unit of staphylocoagulase which is most useful can be defined as the smallest amount in a volume of 0.5 cc (saline diluent) which when added to 0.5 cc of oxalated human plasma (diluted 1:10 with saline) produces a 4+ clot in twenty-four hours or less.

Attempts to obtain sterile staphylocoagulase by other methods reveal some interesting properties. Attempts partially to clear the whole culture by centrifugation caused a marked drop of the staphylocoagulase titer of the supernate, while on fast and prolonged centrifugation the supernate had a very low titer. The finding by Smith and Hale<sup>16</sup> that staphylocoagulase was fairly heat stable was confirmed for both whole cultures and sterile filtrates, but it should be noted that sterile cell-free filtrates are more heat stable than whole cultures. Furthermore, the whole cultures show a great deal of variation in heat stability, varying with different strains and media. It is possible, therefore, to prepare sterile (but not cell free) staphylocoagulase by this method but, because of the total unpredictability of the results as well as the undesirability to altering proteins by heat, staphylocoagulase prepared by this method was not used.

The reported discrepancies in filterability of staphylocoagulase are apparently due to two factors, the nature of the filter and the pH of the culture. None of the bacterial filters are totally satisfactory since they differ only in degree as to how much staphylocoagulase is retained. The best is the Berkefeld V candle, with UF fritted glass (Corning) second best. Berkefeld N, Chamberland L, Mandler, and Seitz filters give culture filtrates which are almost always lacking in staphylocoagulase activity.

The pH of the solution appears at times to influence the filterability of staphylocoagulase through a Berkefeld V candle. Thus, if the pH of the culture is too high or too low no active filtrates will be obtained, in spite of the finding that the

whole culture has a very high staphylocoagulase titer over a wide pH range (5.0-10.0). The cell-free filtrate requires an optimum pH of about 7.2 for maximum activity, and shows only negligible loss of activity within the pH range of 6.0-8.0. However, the finding that an inactive filtrate from a too acid or too alkaline culture when adjusted to pH 7.2 still fails to show staphylocoagulase activity suggests that the failure is in the filtration. Best results require a whole culture at pH of about 7.2 for filtration through a Berkefeld V candle.

Attempts to preserve whole culture preparations by addition of bactericidal and bacteriostatic substances have also revealed some as yet unexplained phenomena. For example, in attempting to sterilize broth cultures with penicillin the interesting effect illustrated in table 1 was noted. Penicillin was added in increasing unit concentrations to aliquots of a 24 hour Tryptose broth culture of *Staphylococcus aureus*. This strain in broth culture was sensitive to a penicillin concentration of

TABLE 1—Effect of Penicillin on the Coagulation of Plasma by a Broth Culture of *Staphylococcus aureus* (Penicillin G) added to aliquots of a 24 hr. broth culture previously diluted 1:20 with sterile saline. 0.5 cc. of sample added to 0.5 cc. of oxalated human plasma diluted 1:10 with saline. Test at 37°C.

Penicillin Concentration units/cc	Subculture	Clotting
0	Positive	4+ in 15 min
10	Positive	4+ in 30 min
20	Positive	4+ in 2 hours
50	Positive	4+ in 4 hours
100	Positive	3+ in 6 hours
200	Positive	Negative
400	Positive	Negative
800	Positive	Negative
1600	Negative	Negative
3200	Negative	Negative

1600 units/cc, but inhibition of coagulation was partial at 100 units/cc and complete at 200 units/cc. In other cases, the reverse could be demonstrated, that penicillin in higher concentrations than that necessary to kill the bacteria could inhibit the coagulating ability. This inhibition has been reported by Mason,<sup>21</sup> while Agnew, Kaplan, and Spink<sup>22</sup> have recorded the same effect with both penicillin and streptomycin.

Crystal violet in a concentration of 1:100,000 shows inhibition of staphylocoagulase activity of an otherwise strongly positive preparation. The same inhibitory effect is shown by high concentrations of phosphate and borate ions.

#### 11. The Evidence for an Accessory Plasma Factor (Coagulase-Globulin) Necessary for Coagulation

If one adds whole culture of staphylococci to oxalated plasma a variable length of time must elapse before the plasma is clotted. This varies from about fifteen minutes to several hours, depending in part on the concentration and activity of the reagents. Since sterile cell-free staphylocoagulase behaves in the same way, this

antihemophilic globulin Tissue extracts contain a considerable amount of the substance in question, as shown by the activity of rabbit testes extract and thromboplastin from rabbit brain Platelets are, interestingly enough, free of

TABLE 2.—*The Distribution of Accessory Factor (Coagulase Globulin CG) in Plasma Fractions Obtained by Alcoholic Fractionation*

Each test contains 2 units of staphylocoagulase 1 per cent of the various fractions and 1 per cent test fibrinogen I 2A 183 37°C. (The plasma fractions were kindly supplied by Dr J T Edsall from the Harvard Fractionation Plant)

Fraction tested	Clotting Time
I 2A 183	No clot
I (392B)	Trace in 4½ hrs.
I (464C)	Trace in 4½ hrs.
II & III	No clot
IV 1	4+ in 3½ hrs
IV 4	4+ in 1½ hrs.

TABLE 3.—*The Relationship of CG (Coagulase Globulin) to Other Globulin Factors*

Staphylocoagulase <sup>1</sup>	Fibrinogen <sup>2</sup>	Globulin Fraction <sup>3</sup>	Clotting <sup>4</sup>
50 units 0.5 cc.	0.5 cc.	—	no clot
50 units 0.5 cc.	0.5 cc.	AcG <sup>5</sup>	no clot
50 units 0.5 cc.	0.5 cc.	V <sup>6</sup>	no clot
50 units, 0.5 cc.	0.5 cc.	I <sup>7</sup>	90 min.
50 units 0.5 cc.	0.5 cc.	Test ext <sup>8</sup>	20 min.
50 units 0.5 cc.	0.5 cc.	Thromboplastin <sup>9</sup>	60 min.
50 units 0.5 cc.	0.5 cc.	Platelet susp <sup>10</sup>	no clot
50 units 0.5 cc.	0.5 cc.	CG <sup>11</sup>	20 min
50 units 0.5 cc.	0.5 cc.	Hem CG <sup>12</sup>	30 min.

<sup>1</sup> Staphylocoagulase Berkeley filtrate containing 50 units in 0.5 cc.

<sup>2</sup> Test Fibrinogen I 2A(183) 2% solution

<sup>3</sup> Globulin fractions as 2% solutions in saline 0.5 cc.

<sup>4</sup> Time required for 4+ clot to form.

<sup>5</sup> Accelerator globulin prepared as outlined by Ware, Guest and Seegers.<sup>14</sup>

<sup>6</sup> Factor V prepared according to Owren.<sup>17</sup>

<sup>7</sup> Harvard Fraction I

<sup>8</sup> Rabbit testes extract 10% suspension in saline.

<sup>9</sup> Thromboplastin Difco standard dilution.

<sup>10</sup> Washed platelets from centrifuged human blood handled with Silicoc coated glassware, resuspended to give a 10% concentration.

<sup>11</sup> Normal Coagulase Globulin from 100% Ammonium sulphate precipitate of normal human plasma.

<sup>12</sup> Hemophilic Coagulase Globulin from 100% Ammonium sulphate precipitate of hemophilic plasma.

activity Since it has so far not been related to any of the other globulin factors it seems justified to consider it as a specialized globulin characterized by its reaction with staphylocoagulase, and to designate it as coagulase globulin or CG

### III Preparation of Crude CG Fraction

Advantage is taken of the observation<sup>17</sup> that the 50 per cent ammonium sulphate fractions contain most of the inhibitor substance. To oxalated human plasma is added slowly and with constant stirring an equal amount of saturated  $(\text{NH}_4)_2\text{SO}_4$  solution. After three hours at 0 degrees C, it is filtered at 0 degrees C and the precipitate discarded. The filtrate is treated with dry  $(\text{NH}_4)_2\text{SO}_4$  to saturation and allowed to stand at 0 degrees C for 6-12 hours. After filtration the precipitate is dissolved in the smallest possible volume of cold saline and dialyzed against several changes of saline for 24 hours at 0 degrees C.

TABLE 4—Clotting Time of Oxalated Plasma with Staphylocoagulase and Coagulase Thrombin after Various Treatment

0.5 cc of oxalated human plasma diluted 1:10 with saline and 0.5 cc of material tested, at 37 C. Clotting time is time required for a 4+ clot to form.

Treatment	Staphylocoagulase	CT
None	30 min.	7 min.
60 C/30 min	30 min.	2½ hrs
Berkefeld V filtration	30 min.	7 min
Sitz filtration	no clot	10 min
Dialysis (24 hr)	30 min	7 min

TABLE 5—Effect of Staphylocoagulase and CT on Purified Fibrinogen and Human Plasma. Fibrinogen fresh 1 per cent solution in saline of Harvard Fraction I 2A run 183 all tests at 37 C and with the same concentration of reagents. Clotting time is time required for the formation of a 4+ clot at 37 C.

1 Fibrinogen + Staphylocoagulase	no clot
2 Fibrinogen + Staphylocoagulase + CG	clot in 1½ hrs
3 Fibrinogen + CT	clot in 7 min
4 Plasma 1:10 + Staphylocoagulase	clot in 30 min
5 Plasma 1:1 + Staphylocoagulase	clot in 4½ hrs
6 Plasma 1:10 + CT	clot in 7 min.
7 Plasma 1:1 + CT	clot in 17 min

### IV Properties of Coagulase Thrombin (CT)

By allowing staphylocoagulase to react with CG at 37 C for an optimum time (usually 2-3 hours), a new substance is formed (coagulase thrombin, CT) which is distinctly different in its properties (table 4).

Staphylocoagulase and CG are little affected by exposure to 60 C/30 minutes, whereas, it is apparent that CT is somewhat heat-labile. None of the three is dialyzable, but there is a striking difference in filterability through bacterial filters, staphylocoagulase being retained by most filters while CG and CT are filterable through all of them. All three are fairly stable even in the crude form at refrigerator temperature.

The most striking evidence that CT is different is its ability to clot fibrinogen free of CG, whereas staphylocoagulase is by itself inactive under this circumstance (table 5). Because of its action on fibrinogen the new substance is designated as



coagulase-thrombin The clotting time of plasma depends on the plasma dilution and the concentration of CT If the clotting times of 1 to plasma are plotted against the concentration of CT (fig 3) a curve is obtained which is similar to figure 1 and to those obtained with thrombin and prothrombin

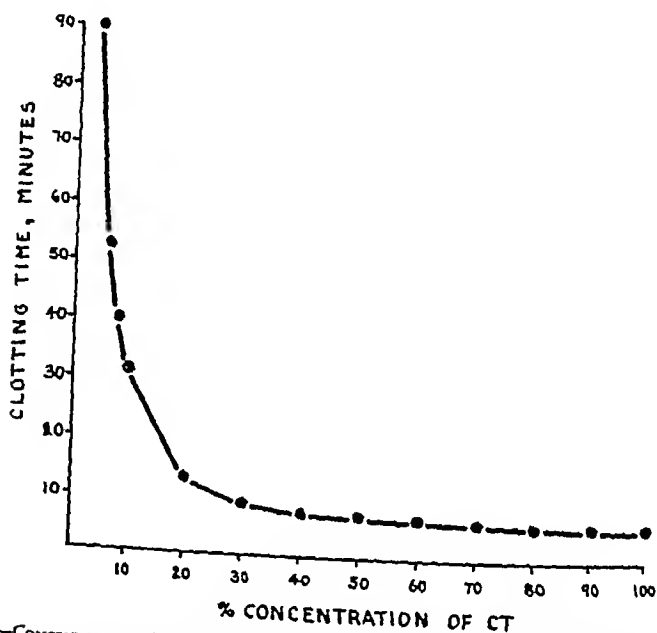


FIG 3—CONCENTRATION-CLOTTING TIME CURVE FOR CT A standard preparation of CT taken as 100 per cent diluted to contain 90 per cent 80 per cent etc. of CT against oxalated human plasma 1 to at 37 C.

#### DISCUSSION

Data has been presented to show that properly prepared filtrates of broth cultures of some strains of *Staphylococcus aureus* contain a substance (staphylocoagulase) which will clot oxalated plasma Staphylocoagulase is by itself incapable of clotting purified fibrinogen, but in the presence of an accessory globulin substance (coagulase globulin, CG) a second agent is formed (coagulase thrombin, CT) whose action is thrombin-like

While reluctant to introduce a new terminology we feel that the one proposed here is the most descriptive and least confusing The literature has been much confused by the application of the term staphylocoagulase or coagulase to whole cultures, filtrates, and as well, to the substance responsible for coagulating fibrinogen Smith and Hale<sup>16</sup> clearly differentiated the two substances, but their suggested terminology of procoagulase for the bacterial substance and coagulase for the agent which clots fibrinogen is based on the assumption that the first is acti-

vated by the globulin substance to form the second. Actually it seems from preliminary observations that the thrombin-like product is derived from the plasma globulin, so that it would not be accurate to call it coagulase. Further justification for our nomenclature will be presented in subsequent studies dealing with the relationship of these substances to prothrombin and thrombin.

The data indicates that CG cannot be identified with either the AcG of Seegers or the V factor of Owren. It appears to be more closely related to anti-hemophilic globulin, but is not identical with it, since CG is obtainable from hemophilic blood as contrasted to anti-hemophilic globulin which is not. The final identification and characterization of CG must await its isolation in purer form than now available, but it promises to shed some additional light on the mechanism of blood coagulation.

### SUMMARY

1. Sterile cell-free filtrates of broth cultures of some strains of staphylococci contain a substance (*staphylocoagulase*) which does not clot purified fibrinogen, but does clot oxalated plasma.

2. When a plasma factor (*coagulase globulin, CG*) is added to staphylocoagulase a thrombin-like substance (*coagulase-thrombin, CT*) is progressively formed which is able to clot purified fibrinogen.

3. When the clotting times of plasma with increasing amounts of either staphylocoagulase or CT are plotted against concentrations of the clotting agents, hyperbolic curves are obtained which are similar to those obtained with classic prothrombin or thrombin.

4. CG appears to be distinct from AcG (Seegers), the V factor of Owren, and "anti-hemophilic globulin" of Taylor and co-workers. The presence of CG in platelets could not be demonstrated.

### REFERENCES

- <sup>1</sup> LOEB, L. The influence of certain bacteria on the coagulation of the blood. *J. Med. Res.* 10: 457, 1903.
- <sup>2</sup> GRATTIA, A. De la coagulation du plasma oxalate par le staphylocoque. *Compt. rend. Soc. de biol.* 2: 1393, 1919.
- <sup>3</sup> ———. Nature et genese de l'agent coagulant du staphylocoque ou staphylocoagulase. *Compt. rend. Soc. de biol.* 3: 584, 1920.
- <sup>4</sup> ———. Influence de milieu sur la production de la staphylocoagulase. *Compt. rend. Soc. de biol.* 3: 585, 1920.
- <sup>5</sup> ———. De la transformation du fibrinogene en fibrine par le staphylocoque. *Compt. rend. Soc. de biol.* 3: 649, 1920.
- <sup>6</sup> ———. La coagulation par le staphylocoque du plasma phosphate. *Compt. rend. Soc. de biol.* 3: 121, 1920.
- <sup>7</sup> GROSS, H. Der Plasmaperinnungstoff der Staphylokokken. *Zentralbl. f. Bakt.* 122: 354, 1931.
- <sup>8</sup> ———. Die Kulturfiltratstoffe der Staphylokokken und deren Beziehungen zueinander. *Ztsch. f. Immunitätsforschung u. Exper. Therap.* 73: 14, 1931.
- <sup>9</sup> ———. Das Plasmaperinnungsphanomen der Staphylokokken. *Klin. Wschr.* 11: 304, 1933.
- <sup>10</sup> GENOUD, O. Contribution à l'étude de l'action du staphylocoque sur le plasma oxalate et sur le fibrinogene. *Ann. Inst. Pasteur* 51: 14, 1933.

- <sup>11</sup> VANBREUSEGHEM R Coagulation et fibrinolyse du plasma et du fibrinogène par le staphylocoque et sa staphylocoagulase *Compt rend Soc de biol* 116 344 1934
- <sup>12</sup> WALSTON H D The clotting of plasma through staphylococci and their products *J Hyg (Cambridge)* 35 549 1935
- <sup>13</sup> LOMINSKI I Preparation of cell free plasma coagulase *Nature (London)* 154 640, 1944
- <sup>14</sup> MUCH H Über einer Vorstufe des Fibrinfermentes in Kulturen von *Staphylokokkus aureus*. *Biochem. Ztschr* 14 143 1908
- <sup>15</sup> CRUKSHANK, R Staphylocoagulase *J Path & Bact* 45 295 1937
- <sup>16</sup> SMITH W, AND HALE J H The nature and mode of action of staphylococcus coagulase. *Brit. J Exp Path* 25 101 1944
- <sup>17</sup> LOMINSKI I, AND ROBERTS G B S A substance in human serum inhibiting staphylocoagulase. *J Path & Bact* 58 187 1946
- <sup>18</sup> KAPLAN, M. H., AND SPINK W W Studies of the staphylocoagulase reaction Nature and properties of a plasma activator and inhibitor *Blood* 3 573 1948
- <sup>19</sup> GERHEIM E. B FERROUSON J H, AND TRAVIS B L. Activation of staphylocoagulase. *Proc. Soc. Exper Biol & Med* 66 525 1947
- <sup>20</sup> MIALE J B AND FRYE J W Standard test for staphylococcus coagulase activity *Am. J Clin Path.* 18 95 1948
- <sup>21</sup> MASON H C. Food poisoning staphylococci and the order of their resistance to penicillin Inhibition of coagulase production *J Immunol* 51 307 1945
- <sup>22</sup> AONEW S KAPLAN, M. AND SPINK, W W Comparative inhibitory effect of penicillin and streptomycin upon the action of staphylocoagulase *Proc Soc Exper Biol & Med* 65 38 1947
- <sup>23</sup> LEWIS, J H. Effect of penicillin on blood coagulation *Proc Soc Exper Biol & Med.* 63 538, 1946
- <sup>24</sup> COHN, E. J STRONO L. E. HUOHES W L JR MULFORD D J, ASHWORTH J N, MILLIN, M., AND TAYLOR, H. L. Preparation and properties of serum and plasma proteins IV A system for the separation into fractions of the protein and lipoprotein components of biological tissues and fluid. *J Am Chem Soc* 68 459 1946
- <sup>25</sup> COHN C. AND WOLFSON W Q Studies in serum proteins I The chemical estimation of albumin and of the globulin fractions in serum *J Lab & Clin Med* 32 1203 1947
- <sup>26</sup> WARE, A. G GUEST, M. M. AND SERGERS, W H Plasma accelerator factor and purified prothrombin activation *Science* 106 41 1947
- <sup>27</sup> OWREN P A The Coagulation of the Blood Investigations on a New Clotting Factor Oslo, J Chir Gundersen Boktrykkeri 1947
- <sup>28</sup> LEWIS J H TAONON H. J DAVIDSON, C. S MINOT, G R AND TAYLOR F H L. The relation of certain fractions of the plasma globulins to the coagulation defect in hemophilia. *Blood* 1 166 1946

## MULTIPLE MYELOMA AS A FORM OF LEUKEMIA

By MICHAEL A. RUBINSTEIN, M D

THE FIRST case of multiple myeloma was reported as *mollities ossium* by Dalrymple, McIntyre, Bence-Jones and Watson<sup>1</sup> in 1848, following closely the first description of leukemia as a disease entity published independently by Craigie, Bennett, and Virchow (1845).<sup>2</sup> The important pathologic studies of Rustizky (1873)<sup>3</sup> and Kahler (1899)<sup>4</sup> established multiple myeloma as a malignant infiltrative disease of the bone marrow of unknown origin, characterized by multiple tumor involvement of the skeleton. Almost at the same time Neumann's<sup>5</sup> description of the myelogenous form of leukemia (1870) and Ehrlich's discovery of blood staining methods<sup>6</sup> led to the recognition of leukemia as a primary proliferative disease of hematopoietic tissues, medullary and extramedullary. Extension of leukemic lesions from the bone marrow to the bone itself was also recorded in early observations (1878).<sup>7</sup>

Since then, as both multiple myeloma and leukemia were recognized as primary infiltrative diseases of bone marrow, their relationship has been the subject of continued discussion. Rustizky<sup>3</sup> was first to classify multiple myeloma as a systemic disease of the hematopoietic tissues related to leukemia, a view taken later by Lubarsch.<sup>8</sup> However, most authors have made a sharp distinction between multiple myeloma and leukemia. The following points have been stressed in the literature and have been conventionally held to distinguish multiple myeloma from leukemia.

### CONVENTIONAL POINTS OF DISTINCTION BETWEEN MYELOMA AND LEUKEMIA

1 *Type of infiltration* (whether circumscribed or diffuse) It has been maintained that whereas multiple myeloma produces circumscribed tumor masses, leukemia is characterized by generalized diffuse infiltration of marrow.

2 *Bone destruction* The distinction made here is that multiple myeloma is characterized by the presence of multiple punched out areas of bone destruction, while in leukemia the infiltrative process in the bone marrow does not as a rule erode the bone cortex.

3 *Visceral involvement* It has been contended that in contradistinction to the myelomatous proliferation which was held to be typically limited to the osseous system, the leukemic infiltration exceeds as a rule the boundaries of bone marrow and is found in visceral organs as well.

4 *Invasion of peripheral blood* While leukemia is manifested, at least at some stage of its evolution, by massive invasion of the peripheral blood by the leukemic cells of the bone marrow, the myeloma cells, it has been maintained, do not pass into circulation.

5 *Biochemical characteristics* Multiple myeloma is associated with abnormalities in protein metabolism manifested in Bence-Jones proteinuria and hyperprotein-

From the Medical Division, Montefiore Hospital, New York, N. Y. The material presented in this paper formed the basis for a Scientific Exhibit in the Section on Internal Medicine at the Ninety-Seventh Annual Session of the American Medical Association, Chicago, Ill., June 23-26, 1948.

emia with hyperglobulinemia. On the other hand, abnormal chemical metabolism in leukemia is manifested mainly in increased basal metabolic rate and elevated uric acid of the blood.

6 *Age incidence* It has been pointed out that while leukemia has been observed at all ages, including infancy, multiple myeloma is a disease of the middle and older age groups.

7 *Symptomatology* Bone lesions are the main basis of the clinical picture in multiple myeloma, while symptoms in leukemia are mainly due to involvement of hematopoietic and visceral organs.

Each of these points in the differential diagnosis between multiple myeloma and leukemia will be discussed. Evidence will be brought to show that the listed points of distinction between multiple myeloma and leukemia cannot be regarded as being of fundamental nature, i.e., there is no sharp demarcation line between the two diseases. Instances are abstracted where cases of multiple myeloma show the various characteristics of leukemia and vice versa.

#### FEATURES OF LEUKEMIA IN MULTIPLE MYELOMA

##### 1 *Diffuse infiltration in multiple myeloma, without circumscribed tumor formation*

It has been shown by many observers that in addition to the well known circumscribed tumor formation, diffuse infiltration of the bone marrow also exists in multiple myeloma. Such cases of diffuse infiltration without any evidence of circumscribed tumor formation are known.<sup>9</sup>

Occasionally cases of multiple myeloma have been observed where the bones appear normal. The spongy trabeculae are numerous and the cortices are not noticeably thinned. The skeletal roentgenograms taken during life in such cases may show at most some diffuse osteoporosis, and do not reveal anything even remotely suggestive of the picture which is accepted as being typical of multiple myeloma. In these cases, only the marrow is modified and replaced diffusely by a tissue which on histologic examination of aspiration material is proved to be myelomatous tissue.

Other cases show some thinning of the cortices as well as great reduction of the spongy trabeculae, but they still may have smooth and undistended bone contour. The roentgenograms show vague mottled rarefaction and thinned cortices. These cases are transitional to the full-fledged picture with multiple areas of bone destruction in typical cases of exuberant growth of myelomatous tissue.

Lack of circumscribed tumor formation does not rule out the possibility of multiple myeloma.

The case reported below is an instance of such diffuse infiltration of bone marrow without apparent evidence of bone destruction. It emphasizes the importance of bone marrow studies in any case of atypical amyloidosis, with or without evidence of bone lesions.

*Case 1* M. S. #38462, white female admitted with signs of renal insufficiency. In 1944, bone marrow aspiration repeatedly revealed from 12 to 27 per cent plasma cells. Soon Bence Jones proteinuria was also noticed.

- Blood examination showed moderate normocytic anemia and occasional plasma cells in the smear. Later a leukemoid picture developed: white blood count 25,000; myelocytes 5 per cent; nonsegmented neutrophils 55 per cent; segmented neutrophils 25 per cent; lymph 3 per cent; plasma cells 1 per cent. Terminally plasma cells increased to 20 per cent. The plasma cells in the blood were morphologically of the same type as those in the bone marrow.

The diagnosis of multiple myeloma was suggested. However, repeated *x-ray* examinations of the skeleton showed no abnormalities at any time, and the serum proteins were low (albumin 3.4 Gm per cent to 4.2 Gm per cent; globulin 1.0 Gm per cent to 2.2 Gm per cent). The Congo red test showed 45 per cent retention. The patient's course was one of rapid deterioration marked by progressive azotemia (BUN up to 150 mg per cent). She died in March 1945.

Autopsy revealed no gross lesions in the skeleton. Microscopic examination of sections of ribs, sternum, vertebrae showed marrow largely replaced by plasma cells; trabeculae thin and amyloid de-



FIG. 1. M. S. X-ray examination revealed no evidence of bone destruction. Most extensive amyloidosis was found, such as perivascular amyloid deposits in the kidney shown here.

posited in walls of the vessels. Amyloidosis was the most important extraskelatal finding. It was most generalized, involving all blood vessels, connective tissue in lungs, ovaries, kidneys, thymus, smooth muscles of most viscera, and the cardiac muscle.

The pathologic diagnosis was plasma cell myeloma, diffuse type, atypical amyloidosis.

## 2. Extraskelatal Visceral Involvement in Multiple Myeloma

Extrasosseous myelomatous infiltrations have been reported in various organs.<sup>10</sup> It is possible that in some cases what may appear to be independent extraskelatal foci might actually have been direct outgrowth of tumor from nearby bones. However, there can be no doubt that in some cases of visceral involvement the infiltrations are of extramedullary origin. Less unusual than grossly discernible foci are microscopic infiltrations in the spleen, kidneys, lungs, lymph nodes.

Autopsy performed by Dr. D. Unterman.

Up to 1936, there were 21 cases of myelomatous visceral involvement recorded in the literature (Blumenfeld). Since that time many other instances have been added. Infiltration of practically every organ in the body has been noted (lungs, heart, spleen, liver, lymph nodes, pancreas, kidneys, adrenals, tonsils, skin, etc). Extraosseous infiltrations are more commonly seen when plasma cells are also found in the blood. Of interest is a case of plasma cell involvement of the tonsils (Jackson et al.) which preceded generalized involvement of bones by many years.

In a recent report<sup>11</sup> visceral involvement in multiple myeloma is presented as a rather common finding in the disease, if carefully looked for in microscopic studies.

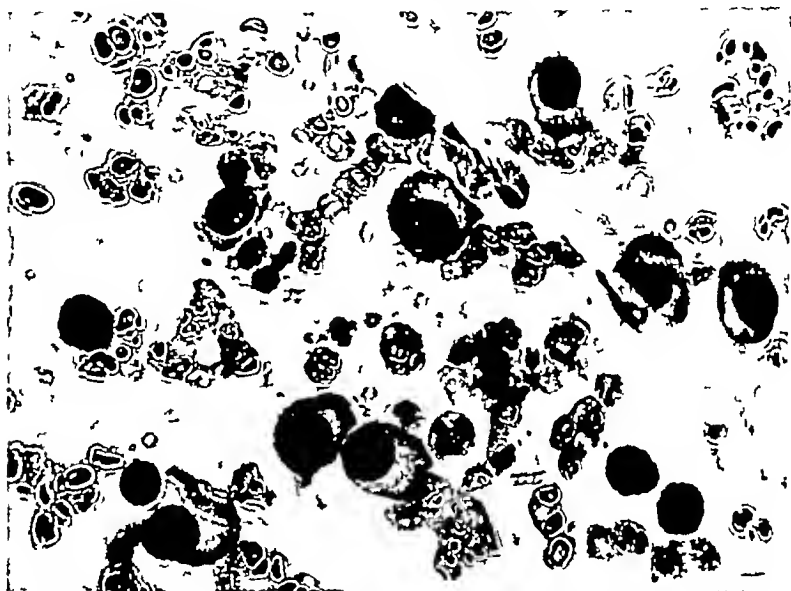


FIG. 2. Bone marrow aspiration in the case of M. S. showing myeloma cell infiltration (diffuse myeloma without bone destruction)

Three of our own cases showed foci of extramedullary myelomatous spread. Two instances with involvement of the tongue (R. S. #362573)<sup>1</sup> and buccal mucosa (M. K. #43608)<sup>12</sup> respectively are reported elsewhere. A third case with extensive extramedullary involvement is presented here.

**Case 2.** A. W. #105534, a 15 year old boy in 1942 gave a three year history of pain in the left hip and fracture of left thigh. X ray studies early in the disease revealed areas of bone destruction in the skull, femur and ribs. Bone marrow aspiration revealed in 1943 the presence of myeloma cells. Bence Jones proteinuria was also discovered. There was no hyperproteinemia. Moderate hypochromic anemia was found.

In the course of a few months new lesions in different bones were discovered and several palpable tumors appeared over the clavicles, sternum, ribs. There were pathological fractures of humerus and lumbar vertebrae. However, the patient's course was marked by spontaneous remissions with healing of

fractures and periods of improved anemia and general condition. Radiotherapy was applied at different sites of the skeleton. Also a course of antimony was given. Treatment was rather difficult to evaluate in view of spontaneous remissions. In 1945 patient developed inability to urinate necessitating an indwelling catheter and ascending urinary infection followed. Terminally there was profuse rectal hemorrhage.

At autopsy\* multiple myeloma was found to involve not only the skeleton but showed also two extensive extraskeletal infiltrations: (1) One tumor mass (800 Gm) involving the pelvic space so as almost



FIG. 3. A. W. multiple punched out areas of bone destruction in the skull, ribs, sternum, vertebrae, humerus, femur, pathological fracture of the hip, palpable tumor formation over a rib.

to obliterate it with compression and infiltration of nearly all pelvic organs (ureters, bladder, prostate, seminal vesicles). (2) Another extraskeletal accumulation of myeloma tissue was found to invade and partially to replace the left kidney which weighed 350 Gm (right kidney weighed 160 Gm).

Microscopically the extraskeletal infiltrations presented the same picture as the myeloma tissue seen in the skeletal lesions. This extraskeletal involvement explained the clinical course marked by renal insufficiency and recurrent pyelonephritis with urinary obstruction. The terminal hemorrhage was due to erosion of the pelvic tumor mass through the perianal structures.

\* Autopsy was performed by Dr. R. Lubliner.





FIG 4 A W Gross specimen of the tumor of left kidney



FIG 5 A W Myelomatous infiltration of kidney (low power)

### 3 *Invasion of Peripheral Blood in Multiple Myeloma*

It has been shown that not only visceral organs, but blood itself may be invaded by myeloma cells. Although massive invasion of peripheral blood, so as to produce

the picture of plasma cell leukemia, occurs rarely (except terminally), occasional myeloma cells may be found quite often (Morissette and Watkins, and others<sup>14</sup>)

In our experience, study of smears made from the white cell layer of packed blood cells facilitates the discovery of occasional myeloma cells, and thus may become an important aid in the diagnosis. We have applied this procedure in multiple myeloma as it is used when looking for occasional abnormal cells in aleukemic forms of leukemia.

The case of M. S. #38462, abstracted previously in this paper as an instance of diffuse myeloma, showed a few myeloma cells in the blood smear. This patient may be designated as aleukemic plasma cell leukemia —by analogy to the con-

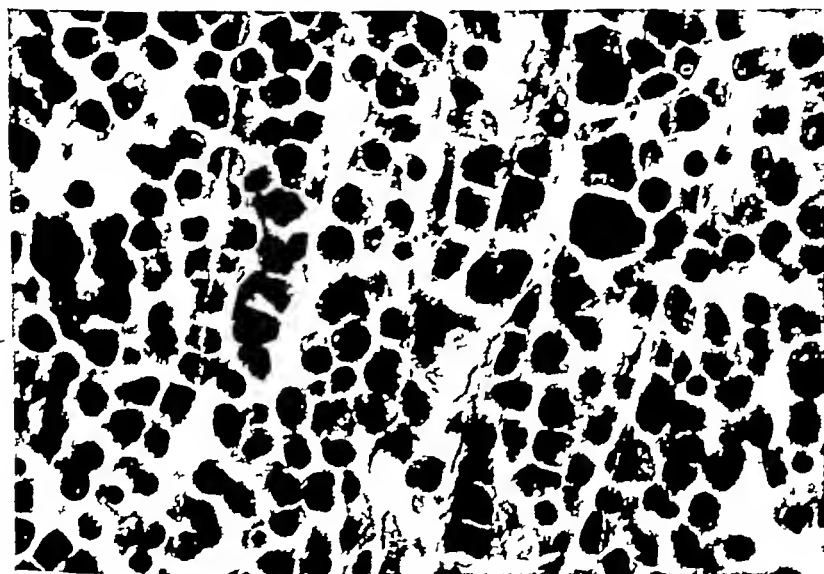


FIG. 6. Section of myeloma tumor of the kidney in the case of A. W. (high power)

ventional leukemia terminology. Another case with massive invasion of the blood, one of plasma cell leukemia, will be briefly abstracted.

*Case 3.* R. S. #362573. In this 58 year old woman the disease was ushered in by a profuse rectal hemorrhage followed by back pain, weakness and soreness of the tongue. On admission there was generalized moderate lymphadenopathy and several red nodules measuring from 1 mm to 1 cm in diameter scattered over the margin of the tongue. There was severe anemia (hemoglobin 31 per cent, red blood count 1,900,000, white blood count 6,100).

Studies of the peripheral blood smear showed 5 per cent plasma cells in an otherwise normal differential count and were the first to suggest multiple myeloma. This diagnosis was confirmed by sternal marrow aspiration which revealed 65 per cent plasma cells. Also aspiration biopsy of the lesions in the tongue showed infiltration by myeloma cells and extramedullary myelomatous spread.

X-ray studies showed multiple punched-out areas in skull ribs long bones. Bence Jones proteinuria was also noticed first intermittently and later constantly. Serum albumin was 2.9 Gm. per cent, globulin 4.4 Gm. per cent.

During eight months observation there was progressive increase of white blood count with simultaneous rise of the number of plasma cells in the peripheral blood. The highest values were white blood count 26,000 with 38 per cent plasma cells 10 per cent myelocytes.

Patient died in renal failure.

#### 4 *Biochemical Characteristics of Leukemia Seen in Myeloma*

The main biochemical findings in leukemia concern the uric acid metabolism and the basal metabolic rate. The uric acid of the blood and the endogenous uric acid elimination are greatly increased in leukemia.<sup>16</sup> Also the basal metabolic rate is increased in the great majority of cases of myelogenous leukemia and in more advanced instances of lymphocytic leukemia, and less frequently in aleukemic



FIG 7 R. S. Concentration blood smear showing myeloma cells

forms of leukemia.<sup>16</sup> These changes are ascribed to the elevated protein catabolism in leukemia.<sup>17</sup>

As may be seen from various reports in the literature, increased uric acid content of blood is a rather common finding in multiple myeloma, probably as frequently seen in this disease as in leukemia.<sup>18</sup> In three of our own cases where uric acid studies were made, it was found to be 5 mg per cent, 5.5 mg per cent and 7.5 mg per cent. As in the case of leukemia, the elevated blood uric content in multiple myeloma is thought to result from the catabolism of the proliferating cells in the bone marrow.

There are also indications of increased basal metabolic rate in multiple myeloma. This appears from some references in the literature,<sup>19</sup> as well as from our own observations. Of 7 cases of multiple myeloma without complications (fever, frac

tures, etc.) in whom we studied the basal metabolic rate, in 5 instances it was found elevated from 25 per cent to 35 per cent

### 5 Age Incidence *Multiple Myeloma in Youth*

The accepted textbook view is that multiple myeloma is a disease of older age. However, review of recent literature will show isolated instances of myeloma in



FIG 8 A W PATIENT IN HIS FIFTH YEAR OF DISEASE

younger age groups, including infants.<sup>20</sup> Also, some older records of bone diseases in youth, originally reported under various descriptions—such as lymphadenia osseum described by Nothnagel<sup>21a</sup>—must be recognized as true myeloma in the light of new knowledge. Especially noteworthy are the cases of myeloma reported by Zach and by Gordon and Schneider<sup>21</sup> in children under 10 years of age.

We have observed a case of multiple myeloma in a child, aged 12 at the onset of disease, in whom the diagnosis was not considered for three years, mainly because of his age. This case (A.W., case 2) showed extensive extraosseous involvement

and has previously been abstracted in this paper as an example of visceral involvement in myeloma. Bone marrow studies were first to suggest the diagnosis of multiple myeloma. Before these studies were done, diagnoses were entertained of osteosarcoma, Schiller-Christian's disease, etc. This case, as well as those reported in the literature, prove that youth should not rule out the possibility of multiple myeloma.

#### 6 *Symptomatology Symptoms of Multiple Myeloma not Referable to Osseous System*

While in the majority of cases, the symptomatology of multiple myeloma is due to tumor involvement of bones with resulting complications (pathologic fractures, deformities, with ensuing pain and neurologic signs, etc.), in a number of patients the complaints are not referable to osseous system,<sup>22</sup> and may be similar to those ordinarily found in leukemia.

In cases of unexplained anemia and cachexia, multiple myeloma is occasionally discovered as the underlying disease. In other instances hemorrhagic manifestations constituted the presenting signs (hematemesis, melena). Epistaxis, ecchymosis, petechiae, and bleeding from gums first suggested leukemia in patients who were proven to have myeloma. Among our own patients, in one instance (A.R. #114881) the clinical picture was dominated by uncontrollable nose bleeds, in another patient (R.S. #362573) the disease was ushered in by profuse rectal hemorrhage.

In other cases, thrombosis was reported as the presenting sign, for example, when failing vision or complete blindness due to thrombosis of central artery of the retina ushered in the clinical picture of multiple myeloma. Gastro-intestinal symptoms (diarrhea, colicky attacks, nausea and vomiting) may dominate the picture. They may be due sometimes to thrombosis of mesenteric vessels. Thrombosis may be due to the increased viscosity of the blood. The latter and the tendency of red cells to clump may give rise to peripheral vascular disturbances not unlike those seen in Raynaud's disease.

Occasionally patients in chronic renal failure diagnosed as nephritis turned out to have multiple myeloma, with the clinical picture dominated by the myeloma kidney.

Very occasionally hepato-splenomegaly was observed in multiple myeloma, and very exceptionally lymphadenopathy. This extraosseous symptomatology in multiple myeloma may be seen not only in the diffuse type of myelomatous infiltrations, but also in cases with circumscribed tumor formation and bone destruction which may remain symptomless for some time. Bone marrow examination will reveal the true nature of the disease in the absence of any symptoms referable to the osseous system.

### FEATURES OF MYELOMA IN LEUKEMIA

#### 1 *Medullary Forms of Leukemia Skeletal Involvement*

Very rare cases of leukemia have been reported (Storti, Klima and Syfried, etc.) where only the bone marrow was involved.<sup>23</sup> No visceral infiltration was found.

even on thorough autopsy examination. In these cases the diagnosis could be made only on the basis of bone marrow studies. These rare forms of leukemia would correspond to the usual forms of multiple myeloma limited to the bone marrow and without visceral involvement.

Less uncommon is involvement of different bones in leukemia. It has been well known ever since Heschl<sup>1</sup> in 1847 described osteolytic lesions in leukemia patients. Bone lesions are more common in acute leukemia, especially in children in whom x-ray examination of the skeleton proved to be an aid in the diagnosis of leukemia.<sup>24</sup> The lesions may take form of tumors, destruction and absorption of bone leading to fractures, periosteal elevations and arthritis<sup>25</sup>, the latter is produced by leukemic proliferation in juxta-articular portions of the bone. Chloroma refers to localized tumors associated especially with the acute forms of leukemia.<sup>26</sup> However, the finding of bone tumors in cases of classic chronic myelogenous leukemia has also been reported.

X-ray studies have shown that in some instances leukemia may lead to generalized decalcification of the skeleton (osteomalacic forms of leukemia). However, occasionally the x-ray pictures of bone infiltration in leukemia may resemble closely those seen in multiple myeloma (Mandl and Saxle<sup>27</sup>).

As an example, the following case is of interest:

*Case 4* F. G. #112875 white female age 30 in 1946 generalized lymphadenopathy and splenomegaly were found. Biopsy of a lymph node as well as peripheral blood and bone marrow studies (90 per cent lymphocytes) were typical of chronic lymphatic leukemia. Patient developed extremely severe pain in left thigh and toes and required increasing doses of opiates. X-ray examination showed areas of translucence in the femur and also several areas of bone destruction in the distal phalanges of the foot. Relief of pain followed x-ray therapy to the affected bones.

## 2. Biochemistry of Leukemia

Bence-Jones proteinuria and hyperproteinemia, admittedly typical of multiple myeloma, have also been occasionally observed in leukemia.<sup>28</sup> These observations were made more frequently in the lymphatic than in the myeloid variety. Magnus-Levy in 1932 collected 11 cases of lymphatic and 5 cases of myeloid leukemia showing Bence-Jones proteinuria. Although only two cases have been reported of leukemia associated with hyperglobulinemia, the actual number is probably much larger, as appears from the literature on occurrence of hyperproteinemia in general. Bence-Jones proteins in the plasma have also been observed in leukemia.<sup>29</sup>

From our own observations, 2 cases of leukemia will be abstracted, where Bence-Jones proteinuria and hyperproteinemia were seen. These will be briefly abstracted. Similar instances were observed by Dr. N. Rosenthal (personal communication to the author).

### *Case 5* Bence Jones proteinuria in leukemia

The diagnosis of lymphatic leukemia in the case of R. K. (case observed on the outside and at Mount Sinai Hospital) a 72-year-old male was made in 1940. At that time generalized lymphadenopathy and hepatosplenomegaly were noticed. Blood examination showed moderate anemia. White blood count was 50,000 with 89 per cent lymphocytes of mature type. Also bone marrow (90 per cent lymphocytes) and biopsy of a lymph node were typical of lymphatic leukemia.

In March 1944 Bence Jones proteinuria was discovered. At that time the hepato-splenomegaly and lymphadenopathy had considerably increased, and white blood count rose to 250,000 with 5% of the lymphocytes. On admission to the Mount Sinai Hospital in March 1944, a rev examination of the bone showed no abnormalities and the serum proteins were normal or low.

At first Bence Jones proteinuria was found only intermittently but since November 1944 it was present constantly in large amounts. During his hospitalization repeated roentgenographic examination



FIG. 9. H. L. LYMPHATIC INFILTRATION OF BONE MARROW IN A CASE OF LEUKEMIA WITH HYPERPROTEINEMIA

were made of the skull, ribs and long bones, but no evidence of bone lesions was ever found. In spite of treatment (radiotherapy transfusions) the patient's course was progressively downhill and he died as the anemia became very severe with very high white blood count (up to 500,000) almost entirely composed of lymphocytes.

#### Case 6 Hyperproteinemia in leukemia.

H. L. #38593 65 year old white male, admitted because of weakness, frequent necklaid, hepatosplenomegaly and general lymphadenopathy. Studies of peripheral blood revealed a picture of leukemia: hemoglobin 6 Gm., red blood count 2,300,000, white blood count 50,000, lymphocytes 6%.

cent This diagnosis was confirmed by sternal marrow examination (90 per cent lymphocytes out of a total nucleated cell count 110 000), and by biopsy of a cervical lymph node

A most striking finding was very marked hyperproteinemia due to hyperglobulinemia, consistently seen on repeated examination

#### Serum protein values

Date	Alb	Glob	Englb	Psglb I	Psglb II
7 26	4 7	9 3	3 1	1 5	4 7
9 20	5 1	8 5	4 5	0 3	3 7

The formol gel test was immediately positive There was excessive rouleaux formation and a rapid sedimentation rate of red cell (80mm/hr) The urine was positive for albumin but negative for Bence Jones protein The basal metabolic rate was plus 21 per cent

X-ray examination of the skeleton failed to reveal any destructive lesion or even osteoporosis

At autopsy the diagnosis of lymphatic leukemia was confirmed

### 3 *Symptomatology of Leukemia Referable to Osseous System*

It has been mentioned in a previous chapter that bone lesions may be found in leukemia, especially in acute forms in children Sometimes symptoms referable to the bones and joints may dominate the clinical picture Pain, limitation of movement and other symptoms which suggest various bone and joint diseases (such as rheumatic fever, Still's disease, caries of the spine, osteomyelitis, etc) may occur in leukemia<sup>30</sup>

Symptoms may arise as the result of compression by bone tumors on nerves Bone tenderness is not infrequent and may be elicited usually in the lower portion of the sternum<sup>31</sup> The following case illustrated the domination of the clinical picture by bone and joint pains

*Case 7* N G #39335, boy of 18, was diagnosed in the beginning as a case of acute rheumatic fever because of migrating pains in the joints of all extremities Later the pain became more localized in the left knee which was swollen and stiff The diagnosis of osteomyelitis was then suggested

However blood and bone marrow studies revealed a picture characteristic of chronic myeloid leukemia Also splenomegaly was soon noticed Throughout the disease, pain in the bones especially in the elbows and knees remained a prominent feature X ray studies revealed generalized decalcification of long bones

### COEXISTENCE OF MULTIPLE MYELOMA AND LEUKEMIA

The literature contains a number of reports indicating the coexistence of leukemia and multiple myeloma<sup>32</sup> Apart from plasma cell leukemia, clinical multiple myeloma was found in combination with lymphatic leukemia and more rarely with myeloid leukemia

Also, experimental evidence suggests a connection between multiple myeloma and leukemia<sup>33</sup> Successive inoculations of transplantable leukemia in mice may give rise to multiple myelomatous infiltrations, instead of true leukemia (Furth)<sup>34</sup> The result obtained, whether multiple myeloma or leukemia, seemed to depend on the dosage of inoculated tissue, and on the state of the recipient animal (whether



previously irradiated or not) In connection with these observations and experiments, our case of extensive combined lymphocytic and plasma cell infiltration is of interest

*Case 8 Combined lymphocytic and plasma cell infiltration*

R. K. #372229 age 50 white female was admitted with a diagnosis of lymphatic leukemia because of marked generalized lymphadenopathy and typical blood findings hemoglobin 6 Gm per 100 cu. cm red blood count 2,000,000 per cu. mm white blood count 18,000 per cu. mm the differential count showed 80 per cent lymphocytes of the mature type 1 per cent plasma cells, 19 per cent neutrophils. Biopsy of a lymph node performed at another hospital was reported as typical of lymphatic leukemia.

At the Montefiore Hospital, bone marrow aspiration showed mixed lymphocytic (38 per cent) and plasma cell (21 per cent) infiltration. The rest of the bone marrow cells of the white and red cell series were crowded out but showed the normal myeloid-erythroid ratio. The mixed lymphocytic and plasma cell infiltration was found on both sternal and iliac bone marrow aspirates. The peripheral blood

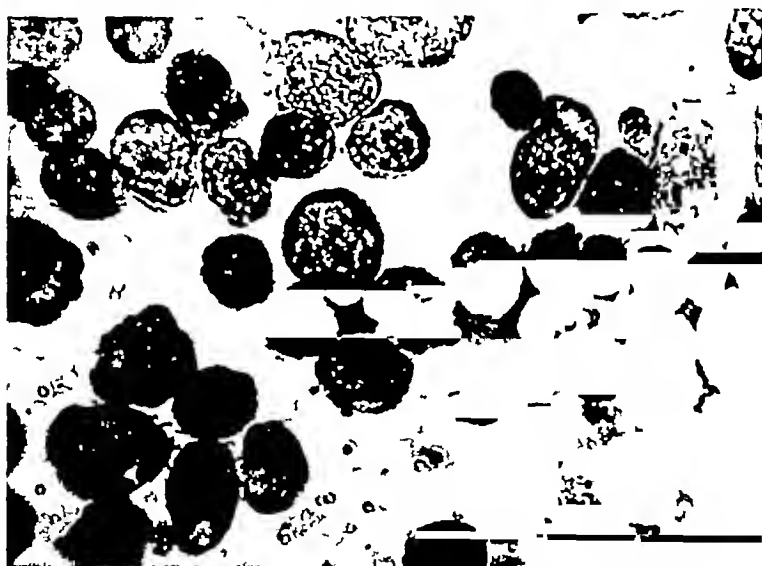


FIG. 11. N. G. BONE MARROW IN STAGE OF ACUTE EXACERBATION OF LEUKEMIA

studies showed white blood count 20,000 lymphocytes 80 per cent, plasma cells 2 per cent. The lymphocytes in the blood and bone marrow were of the small cell type, as seen in chronic lymphocytic leukemia but the plasma cells often showing multiple nuclei with nucleoli were suggestive of myeloma cells.

There was hyperglobulinemia (albumin 2.9 mg per cent, globulin 4.1 Gm per cent) excessive rouleaux formation rapid sedimentation of red blood cells and positive formal gel test of the serum. No Bence Jones proteinuria was seen.

The possibility of multiple myeloma was suggested. However x-ray studies showed no evidence of bone destruction. The patient died four years after onset of disease. Autopsy findings were described as follows:

Malignant mixed lymphocytic and plasma cell lymphoma involving peripheral intrathoracic and intra-abdominal lymph nodes spleen and bone marrow with infiltration of most organs (liver, lungs heart stomach, kidneys, adrenals pancreas) There were both focal and diffuse infiltrations of these organs

The infiltrations, whenever seen were composed of lymphocytes and plasma cells in varying proportions in many places the latter cells being by far predominant and occurring in clumps These plasma cells were described as being identical in cytology and staining reactions (Papanheim stain, etc.) with those seen in plasma cell tumors

The pathologic diagnosis was malignant mixed lymphocytic and plasma cell lymphoma, showing both diffuse infiltration and circumscribed tumor formation

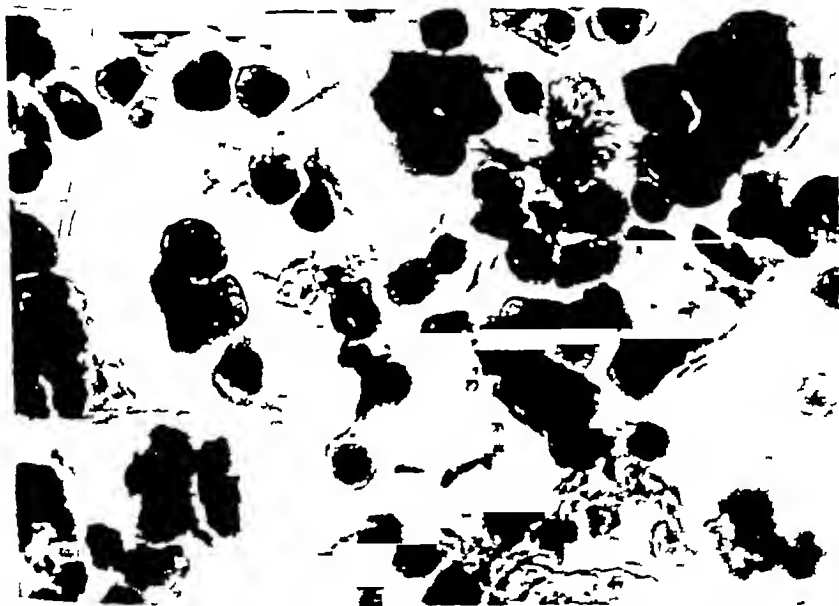


FIG. 11 R. K. HIGH POWER EXAMINATION OF LYMPH NODE SHOWED PREDOMINANTLY PLASMA CELL INFILTRATION Low power examination showed destruction of gland architecture by plasma cell infiltration

#### DISCUSSION

The conventional points in differential diagnosis between myeloma and leukemia have been discussed The following points have been held to distinguish the two diseases (1) Type of infiltration, whether diffuse or circumscribed (2) Bone destruction (3) Extraskelatal visceral involvement (4) Invasion of peripheral blood (5) Biochemical characteristics (6) Age incidence, clinical manifestations

It has been shown, by assembling different data from the literature and on the basis of our own observations, that the difference between myeloma and leukemia, as far as these characteristics are concerned, is merely one of incidence, what is rare in one disease is common in the other Instances of myeloma may show all the characteristics of leukemia, and vice versa, but not with the same frequency

The following table summarizes the discussion

Points in differential diagnosis	Leukemia	Myeloma
Circumscribed tumors	Uncommon	Common
Bone destruction	Uncommon	Common
Visceral involvement	Common	Uncommon
Peripheral blood invasion	Common	Uncommon
Hyperproteinemia	Uncommon	Common
Bence Jones proteinuria	Uncommon	Common
Bone symptoms	Uncommon	Common
Incidence in youth	Common	Uncommon

It is known that there is a difference in incidence of various leukemic characteristics depending upon the cell type of leukemia (myelogenous, lymphatic, etc. Certain features (such as splenomegaly, lymphadenopathy, skin or skeletal involvement etc.) are more common in one variety than in the other. All the features of leukemia may be found in all leukemic varieties, but not with the same frequency. It has been demonstrated in our discussion that myeloma may show all the characteristics of leukemia, and vice versa, but not with the same frequency. This is however merely a quantitative difference. As far as the listed characteristics are concerned, there is no qualitative difference between myeloma and leukemia.

*Myeloma is, then merely a leukemia of plasma cells.* This variety of leukemia is ordinarily of aleukemic type. Moreover, as compared to other leukemias, it is characterized by very common occurrence of bone destruction, by relatively infrequent visceral involvement, and by frequent and characteristic biochemical abnormalities in protein metabolism. It has a definite predilection for older age groups and its clinical picture is usually dominated by bone pathology. In this light viewing myeloma as another member of the leukemia family, it is more plausible to understand the coexistence of myeloma and leukemia as something more than accidental.

#### SUMMARY

The conventional points in the differential diagnosis between myeloma and leukemia have been discussed. Evidence has been brought to show that these points of distinction cannot be regarded as being of fundamental nature. Instances are abstracted where cases of multiple myeloma show the various characteristics of leukemia and vice versa.

1. *Leukemic features in myeloma have been shown in*
  - a. diffuse infiltration in multiple myeloma without circumscribed tumor formation and without any gross bone destruction,
  - b. extraskelatal visceral myelomatous spread involving the kidney, spleen lymph nodes, etc
  - c. invasion of peripheral blood in myeloma—occasional myeloma cells (corresponding to the aleukemic forms of leukemia) may frequently be found in concen-

trated smears, even though they may be missed on routine examination, however, massive invasion of peripheral blood is rare,

d increased uric acid content of the blood and elevated basal metabolism, characteristic of leukemia, frequently seen also in myeloma,

e occurrence of myeloma in youth,

f symptomatology of multiple myeloma at times not referable to the osseous system

2 *Myeloma features in leukemia have been shown in*

a skeletal involvement in leukemia,

b very rare medullary forms of leukemia (without visceral involvement),

c occurrence of Bence-Jones proteinuria or

d hyperproteinemia with hyperglobulinemia in rare cases of leukemia,

e instances when the symptomatology of leukemia was referable to the osseous system

3 *Coexistence of multiple myeloma and leukemia* is reviewed from the literature, and a case is reported of extensive mixed lymphocytic and plasma cell infiltration

In conclusion, the difference between myeloma and leukemia, as far as the listed conventional distinguishing features are concerned, is merely one of incidence what is rare in one disease, is common in the other, and vice versa Multiple myeloma is in all probability a leukemia of plasma cells

#### REFERENCES

- <sup>1</sup> DALRYMPLE J On the microscopic character of mollities ossium *Dublin Quart J M. Sc* 2 85 1848
- McINTYRE, W A case of mollities and fragilitas ossium, accompanied with urine strongly charged with animal matter *Med Chir Tr* 33 211 1850
- JONES H. B On a new substance occurring in the urine of a patient with mollities ossium *Philosoph Tr Roy Soc London* 1 55 1848
- <sup>2</sup> CRAIGIE D Case of disease of the spleen in which death took place in consequence of the presence of purulent matter in the blood *Edinburgh M. & S J* 64 400 1845
- BENNETT J H Case of hypertrophy of the spleen and liver in which death took place from suppuration of the blood, *Edinburgh M. & S J* 64 413 1845
- VIRCHOW R Weisses Blut N Notiz ad Ges d Nat u Heilk 36 151 1845
- <sup>3</sup> RUSTIZKY J Multiple Myeloma *Ztschr Chir* 3 162, 1873
- <sup>4</sup> KAHLER O Zur Symptomatologie des multiplen Myeloms Beobachtung von Albumosurie *Prag Med Wchschr* 14 33 1899
- <sup>5</sup> NEUMANN E. Ein Fall von Leukämie und Erkrankung des Knochenmarkes *Arch d Heilk* 11 1 1870
- <sup>6</sup> EHRICH P Farbenanalytische Untersuchungen zur Histologie und Klinik des Blutes Berlin Hirschwald 1891
- HEISCHL, W Ueber einen Fall von Leukämie *Virchow's Arch f path Anat* 8 353 1854
- NEUMANN E. Ueber myelogene Leukämie *Berl klin Wchschr* 15 69 1878
- <sup>7</sup> LUBARCH O Zur Myelomfrage *Virchow's Arch path Anat* 184 213 1906
- <sup>8</sup> Diffuse Multiple Myeloma Cabor Case 21086 *New England J Med* 212 353 1935
- <sup>9</sup> MICHELSON H E AND LYCH F W Systematized amyloidosis of skin and muscles *Arch Dermat & Syph* 29 805 1934
- ULRICH H Multiple myeloma *Arch Int Med* 64 994 1939
- SNAPPER I Medical Clinics on Bone Disease New York Interscience Publishers Inc 1943
- LICHTENSTEIN L AND JAFFE H L. Multiple myeloma *Arch Path* 44 207 1947

- <sup>10</sup> HALLBERMANN W Zur Kenntnis des primären multiplen Myeloms *Dtschr Arch f Klin. Med.* 163 37 1929
- JACKSON, H PARKER F JR, AND BETHRA J M Studies of diseases of lymphoid and myeloid tissues *plasmacytomas and their relation to multiple myeloma* *Am J M. Sc.* 181 169 1931
- BLUMENFELD, L. Plasma cell tumors of nose and nasopharynx *Ann Otol Rhin & Laryng* 43 436 1936
- DONHAUSER, J L. AND DE ROUVILLE W H Multiple myeloma with special reference to soft tissue metastasis *Arch Surg* 43 946 1941
- NEWSN G R., AND EDWARDS J L. Case of plasma-cell myelomatosis with large renal metastasis and wide-spread renal tubular obstruction *J Path. & Bact.* 58 259 1944
- CHURO, J., AND GORDON, A. J Multiple myeloma with unusual visceral involvement *Arch Path.* 34 546 1942.
- MALLOY, T B Case report *New England J Med* 155 113 1936
- <sup>11</sup> CHURO, J., AND GORDON, A. J Report read before 142nd Annual Convention of Medical Society of State of New York N Y City May 1948
- <sup>12</sup> RUBINSTEIN M. A Plasma cell invasion of peripheral blood in multiple myeloma. *J. Mt. Sinai Hosp.* 12 616 1945
- <sup>13</sup> ——— Use of anemomy in multiple myeloma *Blood*, this issue, p 1068
- <sup>14</sup> MULLER, G L. AND McNAUGHTON, E Multiple myeloma with blood picture of plasma cell leukemia *Folia Haemat* 46 17 1931
- OSGOOD E E. AND HUNTER W C. Plasma cell leukemia *Folia Haemat* 52 369 1934
- PATEK, A J., AND CASTLE, W B Plasma cell leukemia. *Am. J. M. Sc.* 191 788 1936
- MALLOY T B Case records of Massachusetts General Hospital *New England J Med* 112 28, 1935
- PINEY A AND ROUGH J S Multiple myeloma aleukemic and leukemic *Folia Haemat.* 41 37 1937
- MORISSETTE, L. AND WATKINS, C. H Multiple myeloma. *Proc. Staff Meet. Mayo Clinic* 17 433 1942.
- ASKANAZY, M. AND DUBOIS-FERRIERE, H. La cellule plasmatique et la leucemie plasmacellulaire *Helv Med Arch* 9 427, 1942.
- <sup>15</sup> JUGENBURO A., AND TCHOTCHIA K. Neue Ergebnisse zum Verständnis des Leukämieverlaufs. *Strahlentherapie* 41 86, 1931
- <sup>16</sup> RIDDLE M. C. AND STURGIS C. C. Basal metabolism in chronic myelogenous leukemia *Arch. Int. Med* 39 255 1927
- KRANTZ C. Q., AND RIDDLE, M C The basal metabolism in chronic lymphatic leukemia *Am. J. M. Sc* 175 229 1928
- <sup>17</sup> BALBRIDGE C. W., AND BAKER A. Relationship between oxygen consumption and nitrogen balance in leukemia *Arch Int Med* 51 589 1933
- <sup>18</sup> STEWART A AND WEBER F P Myelomatosis *Quart. J Med* 7 211 1938
- <sup>19</sup> WINTROBE M. M. Clinical Hematology, ed 2. Philadelphia Lea and Febiger 1947
- <sup>20</sup> SLAVENS J J Multiple myeloma in child *Am. J. Dis Child* 47 821 1934
- LAURENTIUS P Multiples Myelom in Kindesalter *Monatschr f Kinderh* 73 95 1938
- GHORMLEY R. K. AND POLLOCK, J A. Multiple myeloma *Surg., Gynec. & Obst.* 69 64 1939.
- WILLIAMS G D RITCHIE, W A., AND TITERINGTON P F Multiple bony lesions suggesting myeloma in pre-Columbian Indian aged 10 years *Am J Roentgenol* 46 351 1941
- <sup>21</sup> ZEH K. Über Myeloma in Kindlichen Alter *Virchows Arch f path Anat* 238 310 1932.
- GORDON H AND SCHNEIDER B Plasma cell myeloma in a child *International Clinics* 4 173 1940.
- <sup>21a</sup> NOTHNAOEL Lymphadenomia Ossium *Festschrift für R. Vorchow*, 1891
- <sup>22</sup> STEWART A AND WEBER F P Myelomatosis *Quart J Med* 7 211 1938
- ULRICH H. Multiple myeloma *Arch Int Med* 64 994 1939
- <sup>23</sup> STORT E Über einen Fall von lymphatische Leukämie mit ausschliesslicher Lokalisation im Knochenmark und ueber die Bedeutung der Sternalpunktion für die Diagnose dieser Krankheitsform. *Dtsch Arch Kl Med* 180 612, 1937

- KLIMA R., AND SYTRIED H. Lymphatische Leukämien unter dem Bilde der thrombopenischen Purpura hamolytischen bzw aplastischen Anämie, und Agranulocytose Wien Arch inn Med 30 1 1937
- <sup>24</sup> SILVERMAN F N The skeletal lesions in leukemia Am J Roentgenol 59 819 1948
- <sup>25</sup> PATRASSI, G Zerstörungsvorgänge am Skelett in Verlauf leukämische Erkrankungen Beitr z path Anat u z allg Path 86 643 1931
- BATT J M. AND VOGT, E C Bone changes in leukemia in children Am J Roentgenol 34 310, 1935
- GRAVER L F, AND COPELAND M M Changes of the bones in the leukemias Arch Surg 30 639 1935
- APITZ K. Die Leukämien als Neubildungen Virchows Arch f path Anat 299 1, 1937
- TOWNSEND, S R. A single myeloid bone tumor associated with a blood picture of chronic myelocytic leukemia. Canad M A J 40 352, 1939
- WINTROBE M M., AND MITCHELL, D M Atypical manifestations of leukemia Quart J Med 9 67, 1940
- <sup>26</sup> BRANNA D Chloroma Bull Johns Hopkins Hosp 38 189 1926
- <sup>27</sup> MENDLE, K, AND SAXL, O Bone changes in leukemia Am J Roentgenol 44 31 1940
- <sup>28</sup> MAGNUS-LEVY A Ueber die Myelomkrankheit vom Stoffwechsel die Bence Jones Proteinuria Ztschr f Klin Med 119 307, 1932
- BÜCKEL, H Beitrag zur Kenntnis der Hyperproteinämie Ztschr f Klin Med 127 552, 1934
- <sup>29</sup> KEILBACK, H Die Hyperproteinämie und Hyperglobulämie, als Symptome einer chronischen akuten kachemischen Myelose Folia Haemat 55 406 1936
- <sup>30</sup> SMITH, C H Leukemia in childhood with onset simulating rheumatic disease J Pediatr 7 390 1935
- EHRLICH J C AND FÖBER, S Periosteal ossification in myelogenous leukemia Arch Int Med 53 938 1934
- <sup>31</sup> CRAVER, L F Tenderness of the Sternum in Leukemia Am J M. Sc. 174 799 1927
- <sup>32</sup> ELLERMANN V Ein Fall von Myelom mit myelose der Organe und leukämische Blutveränderung Ztschr f allg Path u Path Anat 31 33 1923
- HALLERMANN W Zur Kenntnis des primären multiple Myeloms Dtsch Arch f Klin Med 165 57 1929
- SNAPPER I Medical Clinics on Bone Diseases New York, Interscience Publishers 1943
- <sup>33</sup> KREBS C., RASK NIELSEN H C., AND WAGNER A Radiosensitivity of lymphosarcomas Acta Radiol 11 487, 1930
- FURTH J AND STRUMIA, M Studies on transmissible lymphoid leucemia of mice J Exp Med 53 715, 1931
- RICHTER M. N AND McDOWELL E C Studies on mouse leukemia J Exp Med 57 1 1933
- KAALUND-JØRGENSEN O Experimental studies on a transmissible myelomatosis Acta Radiol Suppl 29 1936
- <sup>34</sup> FURTH J Transmission of myeloid leukemia of mice its relation to myeloma J Exp Med 61 423 1935

## USE OF ANTIMONY IN MULTIPLE MYELOMA

By MICHAEL A. RUBINSTEIN, M.D.

IN A previous communication<sup>1</sup> the rationale of antimony treatment in multiple myeloma was discussed, and a preliminary report was given on the results obtained. Because the therapeutic effect of antimony is largely confined to diseases associated with hyperglobulinemia regardless of the etiologic factor involved (kala-azar, lymphogranuloma venereum, schistosomiasis, etc.), it was assumed that the drug might be found effective in another disease with hyperglobulinemia namely, in multiple myeloma. The effects of antimony as originally reported were to induce increased radiosensitivity, and to result in certain alterations in the morphology of the plasma cells.

The present paper, based on a total of 11 cases, treated with antimony, reports some new observations in addition to those mentioned in the first communication. These observations concern the effect of antimony treatment seen in (1) stoppage of uncontrollable bleeding, (2) reduction of hyperglobulinemia, (3) regression of palpable tumors. The dosage and the preparations used were the same as those previously described.<sup>1</sup>

### 1. Relief of Uncontrollable Bleeding

The patient A. R., #114881, was a 64 year old male. In July 1945 he developed recurrent severe nosebleeds and soon afterward started to complain of backache. The nosebleeds were stopped at that time by cauterization. In January, November and December 1946 and early in 1947 there was a recurrence of epistaxis controlled by packing. In April 1947 the nosebleeds became almost continuous and uncontrollable by either packing or cauterization and patient had to be hospitalized.

Sternal bone marrow aspiration in May 1947 revealed a picture of plasma cell multiple myeloma. Because of uncontrollable nosebleeds frequent blood transfusions were necessary.

On admission to Montefiore Hospital in January 1948 the patient was bleeding profusely from both nares but no specific bleeding points could be seen. There was gibbus deformity in the mid-thoracic area and pain with rootlike distribution over D-7. The liver was enlarged four fingerbreadths below the right costal margin.

There was severe anemia (hemoglobin 42 per cent, red blood cells 2,000,000) the white blood cell count was 5,000 with a normal differential picture. The platelet count was normal (260,000) as was the clotting and bleeding time. The only abnormality indicative of a hemorrhagic tendency was the failure of the clot to retract after forty-eight hours.

Sternal and iliac bone marrow examination showed about 70 per cent plasma like myeloma cells.

The total serum protein was 12.3 Gm, albumin 1.1 Gm, globulin 11.2 Gm, the blood urea nitrogen was 22.1 mg. per cent.

X-ray examination revealed numerous well circumscribed osteolytic areas in the vault of the skull, in the upper ends of both femurs, in the clavicles and a destructive lesion of D-9 with partial collapse of the vertebral body.

When in spite of repeated cauterizations, packing and transfusions, the nosebleeds continued to be

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From the Medical Division, Montefiore Hospital, New York, N. Y. The material presented in this paper formed in part the basis of a Scientific Exhibit in the Section on Internal Medicine at the 97th Annual Session of the American Medical Association in Chicago, Ill., June 23-28, 1948.

profuse radium therapy to the nasal mucosa was applied. Small catheters containing radium were inserted alternately in both nares on January 16 and 20, 1948. However, no improvement in the bleeding tendency was noted.

A course of antimony treatment was begun on February 23 and completed on March 17, 1948. Fifteen Gm. of neostibosan were given intravenously divided in 48 doses. Progressive diminution of bleeding

TABLE 1—Serum Protein Values

Case	Date	Albumin	Globulin
D. C.			
Neostibosan started 4 7 46	4 5 46	3 1	8 7
	completed 6 18 46		
	5 17	3 1	6 8
	7 3	2 5	5 8
	8 14	4 7	6 9
Second course of neostibosan started 8 16	completed 10 22		
	9 6 46	2 1	4 9
	1 20 47	2 0	5 3
Third course of antimony started 2 18	completed 3 20		
	3 24	2 1	5 9
	4 16	1 9	7 6
A. L.	9 9 46	4 0	5 2
Antimony started 9 25	completed 10 20		
	11 13	2 8	4 2
	12 6	3 4	4 2
Second course started 1 15 47	discontinued 1 25 47		
	2 16	3 6	3 5
A. R.	1 9 48	1 1	11 2
	2 13 48	1 4	11 1
Antimony started 2 16 48	completed 3 17		
	2 18	1 1	10 1
	3 19	0 8	8 6
	5 7	1 5	9 7
Second course started 6 11	discontinued 6 27		
	6 19	1 9	8 5
	7 14	1 5	9 9
	8 4	1 2	9 8
Third course started 8 10	completed 9 10		
	8 27	1 4	9 7
E. E.	1 12 48	2 4	7 1
	2 4	2 4	8 1
Neostibosan started 2 10 48	completed 3 5		
	3 10	2 4	5 4
	5 7	1 6	6 5

The case histories of D. C. and A. L. are to be found in the first communication<sup>1</sup> and those of A. R. and E. E. in this paper.

was observed in March 1948 followed by complete cessation at the beginning of April 1948. Except for a single episode of nosebleed on June 25, 1948 and occasional oozing in August 1948, there was no recurrence of bleeding to October 1948 (time of writing). Both episodes of bleeding were promptly controlled by a repeated course of neostibosan.

Since the beginning of April 1948 no nasal packing has been necessary and the patient was discharged in October 1948.

Blood examination revealed reduction of hyperglobulinemia (table 1).



## 2. Reduction of Hyperglobulinemia

In reviewing the serum protein data of all patients treated, it was found that in all 4 cases with marked hyperglobulinemia there was some reduction of the latter following antimony treatment.

A slow rise of serum globulin was observed a few weeks after discontinuance of treatments. However, it dropped again following repeated courses of treatment.

There were no significant changes in serum protein in three cases without hyperglobulinemia. In four cases the serum proteins were not followed during treatment.

## 3. Regression of Palpable Tumors

Disappearance of palpable tumors following antimony treatment was observed in all three cases showing such lesions.

Two cases were previously reported.<sup>1</sup> In one of them (A. W. #105534) rapid disappearance of external bone tumors could not be attributed with certainty to the drug, since spontaneous regression was observed in this patient. The results obtained in the second case (D. C. #110504) were more conclusive. Two tumor masses had been noticed by the patient two years prior to admission and were progressively increasing in size, in spite of x-ray treatment. At the completion of neostibosan injections, and before further x-ray treatment, the visible tumors were found to be greatly reduced and became hardly noticeable. They completely disappeared soon after the subsequent x-ray therapy.

A third case since observed will now be described, where rapid and complete disappearance of multiple palpable tumors followed antimony treatment without any local x-ray treatment.

M. K. #43608, a 61 year old female, admitted on 10.2.1947, gave a history of pain in the back of 6 months duration. Several masses on the forehead were noticed five months prior to admission and they were progressively increasing in size.

On examination four palpable masses were found over the scalp: a midfrontal mass measuring 4 cm. x 3 cm. x 2 cm., a latero-frontal 3½ cm. x 3 cm. x 1½ cm., a fronto-temporal 3 cm. x 3 cm. x 2 cm. and a parietal mass 4½ cm. x 3 cm. x 3 cm. These masses were fixed to the underlying tissues, not movable, nontender.

X-ray examination revealed numerous areas of destruction in the entire cranium as well as in the mandible, humerus, scapula, clavicles and ribs. Blood studies showed severe anemia (hemoglobin 7.7 Gm./100 cu. cm., red blood cells 1,920,000), white blood cells 15,000 with normal differential count. Blood urea nitrogen was 61 Gm. per cent, total serum protein 8.2 Gm. per cent, albumin 3.5 Gm., globulin 4.7 Gm.

Sternal and iliac bone marrow aspirations revealed clumps of myeloma cells. The diagnosis of multiple myeloma was confirmed by the presence of Bence Jones proteinuria. Aspiration of the visible masses on the forehead showed myelomatous infiltration.

A course of neostibosan treatment was started on 10.11.47. Practically complete disappearance of all four visible tumors was noticed after two weeks.

However, the patient's course was progressively downhill with increasing azotemia. Because of the severe backpain, x-ray therapy was applied to the lower spine, but at no time was radiotherapy given to the frontal tumor masses.

Patient died in uremia (blood urea nitrogen rose terminally to 186 mg. per cent) on 12.5.47.

Autopsy findings showed multiple myeloma of the skeleton with extension of myeloma tissue from the cranium to the scalp and cerebral dura. The kidneys presented the picture of myeloma nephrosis.

When recently yet another case of multiple myeloma (E. E. #457079) with large frontal palpable masses was treated with neostibosan, no regression of the

masses was obtained. A biopsy was performed and showed that these masses consisted not of myelomatous tissue but of amorphous (amyloid?) mass. At the time



FIG. 1. VISIBLE TUMOR MASSES IN THE SKULL BEFORE TREATMENT



FIG. 2. DISAPPEARANCE OF THE VISIBLE TUMOR MASSES TWO WEEKS AFTER A COURSE OF NEOSTIBOSAN INJECTIONS

when treatment was started in this 52 year old patient he was completely immobilized in bed because of multiple pathologic fractures and most extensive involvement of the skeleton. There was no noticeable effect of the single course of

treatment on the clinical course, but a reduction of hyperglobulinemia was noticed (table 1)

### COMMENT AND SUMMARY

The effect of antimony treatment (neostibosan) of multiple myeloma is described. The following observations are reported

1 *Control of bleeding* In one instance of multiple myeloma the presenting symptom was uncontrollable nosebleed of one and one-half years' duration. The platelet count as well as the clotting and bleeding time were normal, the only abnormality was the failure of the clot to retract. It is possible that the latter abnormality was connected with the abnormal protein composition of the blood (hyperglobulinemia was found).\*

Topical treatment, including repeated cauterizations and radium application to the nasal mucosa, remained without any effect, and constant packing and frequent transfusions were necessary. Following a course of neostibosan injections there was gradual diminution of the bleeding, and after one month complete cessation of bleeding was noted. Since that time (6 months at the time of writing) the bleeding did not recur, and the patient was discharged from the hospital. At the same time a moderate decrease of hyperglobulinemia was observed.

2 *Reduction of hyperglobulinemia* In reviewing all other cases treated it was found that in all four instances with hyperglobulinemia there was reduction of the serum globulin content. When a few months later the hyperglobulinemia was rising, a repeated course of antimony was followed again by its reduction.

In three instances without hyperglobulinemia no change of serum globulin was noted following the antimony treatment. In four other cases the globulin changes were not followed.

3 *Regression of palpable tumors* Three instances with visible tumors, a rather uncommon phenomenon in multiple myeloma, were observed.

Disappearance of the palpable tumors in two patients after combined antimony and radiotherapy was reported in a previous communication. In the present paper, almost complete disappearance of palpable tumors following antimony treatment alone without radiotherapy is reported.

With regard to these observations, the following reservations should be kept in mind

1 In evaluating the influence of antimony on multiple myeloma it is necessary to realize the possibility of occasional spontaneous remissions in this disease, as well as of a prolonged course over a period of years with relative freedom from symptoms, and occasional sensitivity to radiation.

2 The number of observations is insufficient to warrant at this point conclusions as to the *therapeutic value* of antimony. They indicate merely a possible influence of antimony on the myeloma tissue and on the disturbed chemistry of myeloma.

### REFERENCES

- <sup>1</sup> RUBINSTEIN M. A. Chemotherapy of multiple myeloma. The use of antimony. *Blood* 2: 555 1947
- LICHTENSTEIN L. AND JAFFE H. L. Multiple myeloma. *Arch. Path.* 44: 207 1947
- LENER A. B. AND WATSON C. Y. Studies of cryoglobulinemia (Unusual purpura associated with the presence of a high concentration of cryoglobulinemia). *Am. J. M. Sc.* 214: 410 1947

# ABSTRACTS

JOSEPH F. ROSS, M.D., *Editor*

## ABSTRACTERS

HELEN W. BELDING, M.D., Winston-Salem, N.C.  
SHEILA T. CALLENDER, M.D., Oxford, England  
GEORGE E. CARTWRIGHT, M.D., Salt Lake City  
ROGER C. CRAFTS, Ph.D., Boston  
CHARLES P. EMERSON, M.D., Boston  
SOLOMON ESTREN, M.D., New York  
CLEMENT A. FINCH, M.D., Seattle

OLIVER P. JONES, Ph.D., Buffalo  
CONRAD MAIER, M.D., Zurich, Switzerland  
MILOŠ NETOUSEK, M.D., Prague, Czechoslovakia  
JEAN P. SOULIER, M.D., Paris  
RAMÓN M. SUAREZ, M.D., San Juan, Puerto Rico  
WM. N. VALENTINE, M.D., Pacific Palisades, Cal.  
JAN WALDENTRÖM, M.D., Uppsala, Sweden

## LEUKEMIA AND MALIGNANT LYMPHOMA

**LEUKEMIA IN CHILDHOOD. A CLINICAL AND ROENTGENOGRAPHIC STUDY OF SEVENTY-TWO CASES.** *J. H. Dale, Jr.* From the New York Hospital and the Departments of Pediatrics and Radiology, Cornell University Medical College, New York, N.Y. *J. Pediatr.* 34: 421-432, 1949.

The clinical and roentgenologic findings in 72 children with leukemia have been analyzed. X-ray evidence of bone involvement was obtained in over 70 per cent of cases studied, by far the most common abnormality being the appearance of a transverse band of diminished density in the metaphyses of the long bones. This band was the first sign of skeletal disease in the majority of patients and the sole osseous change in almost half of the cases exhibiting x-ray changes. Roentgenographic signs when present invariably included evidence of involvement in the knee area, in view of which it is suggested that routine roentgen examination of this region might provide a helpful screening technique in the investigation of all suspected cases of leukemia, perhaps obviating the necessity of more extensive skeletal surveys.

C. P. E.

**CHRONIC LEUKEMIA OF LONG DURATION. A REPORT OF 31 CASES WITH A DURATION OF OVER 5 YEARS.** *H. C. Moffitt, Jr. and J. H. Lawrence.* From the Divisions of Medical Physics and Medicine, University of California, Berkeley, Calif. *Ann. Int. Med.* 30: 778-790, 1949.

The life duration of patients with chronic leukemia is summarized from the literature by the authors. The frequency of remission, effect of infection and treatment is discussed. The hematologic data of 31 patients with lymphogenous and myelocytic leukemia chosen from 190 cases of the authors because of their long survival are tabulated.

C. A. F.

**OBSERVATIONS IN GUINEA PIGS FOLLOWING INJECTION OF SPECIFIC HEMATOPOIETIC SUBSTANCES DERIVED FROM BEEF LIVER.** *L. M. Meyer and A. Sawitsky.* From the Department of Therapeutics, New York University College of Medicine, New York, N.Y. *Am. J. Path.* 24: 835-855, 1948.

The authors report pathologic changes observed in guinea pigs receiving repeated intramuscular injections of extracts prepared from normal beef liver as follows:

An ethanol extract of dried liver, after concentration and saponification, was repeatedly extracted with ether before and after treatment with carbon dioxide and hydrochloric acid. After successive extractions with petroleum ether and methanol, the material was treated with lead and the acidified ether-insoluble lead salts were crystallized at minus 20 degrees C. in acetone. The noncrystalline material in the mother liquor having been concentrated (B-acids), it was separated by succination into carbinols and noncarbinols.

Nine animals received B-acid extracts derived from 200-800 grams of beef liver. Seven of this series

developed Hodgkin's like lesions in cervical nodes, spleen, liver, adrenal and kidney. The bone marrow exhibited myeloid hyperplasia in 6, lymphoid hyperplasia in 1 and no changes in 2 animals. Myeloid metaplasia of the cervical nodes and spleen were noted in 1 guinea pig. The carbinol fraction was injected into 12 guinea pigs, in 9 of which a lymphoid reaction developed, myeloid changes being noted in another of this series. Of 18 guinea pigs receiving the noncarbinol fraction, 13 exhibited varying grades of myeloid reaction, 5 others presenting evidence of a mixed myeloid and lymphoid stimulation, definite myeloid hyperplasia resulted in 13 animals of this series.

It is concluded that beef liver extracts of the carbinol type stimulated lymphoid hyperplasia and infiltration and those of the noncarbinol type myeloid hyperplasia and infiltration when injected into guinea pigs. The resultant lesions were clinically and pathologically dissimilar to spontaneous leukemia. A reciprocal relationship between myeloid and lymphoid tissues with respect to their reactivity to hemopoietic stimulation and relative rates of proliferation appears to have been confirmed.

C. P. E.

**OBSERVATIONS IN GUINEA PIGS FOLLOWING INJECTION OF SPECIFIC HEMATOPOIETIC SUBSTANCES DERIVED FROM URINES OF HUMAN LEUKEMIC SUBJECTS.** *A. Sawitsky and L. M. Meyer*. From the Department of Therapeutics, New York University College of Medicine, New York. *N. Y. Am. J. Path.* 44: 1117-1137, 1948.

Guinea pigs were injected with urine extracts including the carbinol fractions of urines from patients with lymphoid leukemia, and noncarbinol fractions derived from the urine of patients with myeloid leukemia. Although the resultant lesions were clinically and pathologically dissimilar to those characteristic of spontaneous leukemia, the results suggested that the urines of leukemic patients contain materials which are extractable and separable by the methods described. Carbinol fractions inducing a specific lymphoid hyperplasia and infiltration, and the noncarbinol fractions inducing a myeloid response.

C. P. E.

**HODGKIN'S DISEASE. A CLINICAL-PATHOLOGICAL REVIEW OF ONE HUNDRED FIFTY CASES.** *W. L. Bessell*. From the Department of Pathology, University of California Medical School, San Francisco, Calif. *California Med.* 70: 87-92, 1949.

In 150 cases of Hodgkin's disease studied during the last twenty-five years at the University of California Hospital, the average survival period was forty-one months. Individuals whose lesions histologically were paraneoplasia had no longer survival periods than those with the classic granuloma. The authors emphasize that non-Hodgkin's disease tumors are more likely to be included as Hodgkin's sarcoma or as paraneoplasia than they are to be confused with the granulomatous form. In this series, the tuberculin test was negative in a high percentage of individuals studied. The incidence of tuberculosis associated with the Hodgkin's was only slightly higher than in the general autopsy group. Bacteriologic studies were unproductive except that fertile egg passage of cell-free lymph node filtrates resulted in increased egg mortality and increased cutaneous sensitivity reactions to the harvested amniotic fluid.

W. N. V.

**HODGKIN'S DISEASE. A HISTOPATHOLOGICAL AND CLINICAL CLASSIFICATION WITH RADIOTHERAPEUTIC RESPONSE.** *P. F. Salyers and S. J. Eisenberg*. From the Departments of Surgery and Radiology, Medical College of Virginia, Richmond, Va. *Am. J. Roentgenol.* 61: 369-379, 1949.

Hodgkin's disease is classified into (1) compact cellular, (2) fibrogranulomatous and (3) loosely cellular types. The compact cellular type was characterized by closely packed lymphocytes, some reticulo-endothelial proliferation with Sternberg-Reed cells. The fibrogranulomatous type was that of typical Hodgkin's granuloma. The loosely cellular type showed complete loss of structure of the lymph nodes with sheets of reticulo-endothelial cells and frequent mitotic figures. The report is concerned with an attempt to prognosticate from the histologic picture of 24 patients the clinical course of the disease. The compact cellular type had a life expectancy of 48 to 160 months, the fibrogranulomatous 20 to 60 months and the loosely cellular 12 to 20 months.

The prognostic implication of these studies would seem rather similar to the more extensive studies

of Parker and Jackson. It is unfortunate that some differences in classification make it impossible to compare the different groups of patients.

C A F

**SKELETAL LESIONS IN HODGKIN'S DISEASE.** *E. H. Falconer and M. E. Leonard.* Department of Medicine, University of California Medical School, San Francisco, Calif. *Ann Int Med* 29: 1115-1131, 1948.

In summarizing their own and other reported material on skeletal lesions of Hodgkin's disease, the authors stress the underlying marrow involvement. They have studied by sternal aspiration 59 patients with proven Hodgkin's disease and reviewed pathologic material on 20 patients looking for marrow involvement. One is impressed by the nonspecificity of changes in differential cell counts of the marrow which include (1) a shift to the left in neutrophil series with a relative decrease in segmented neutrophils and an increase in bandforms, (2) increase in eosinophilic myelocytes. There also appeared to be increased megakaryocytes in several instances. It would have been interesting to determine how frequently a specific diagnosis could have been made on aspirated pieces of marrow prepared by fixation and sectioning. In this respect, the authors indicate only that 10 of 11 of 20 autopsied cases of Hodgkin's disease was found in the marrow sections reviewed. The difficulty of morphologic differentiation between reticulo-endothelial cells, Sternberg-Reed cells and megakaryocytes is not discussed.

C A F

**DIAGNOSIS OF PRIMARY HODGKIN'S DISEASE OF THE STOMACH.** *E. L. Parker and S. M. Roberts.* From the Department of Radiology, University of Louisville School of Medicine, Louisville, Ky. *Radiology* 52: 75-78, 1949.

To the 22 cases of primary Hodgkin's disease already in the literature, the authors add one of their own. Interest in this diagnosis stems from the fact that so far as published reports go, if the patient survives the operation of gastrectomy, complete cure is likely. The usual preoperative diagnosis, based on x-rays, is extensive infiltrating carcinoma of the stomach. The authors suggest, however, that the characteristic of Hodgkin's disease is polyp-like masses along the involved gastric area on the x-ray, an appearance which is relatively constant in all these cases, although there may be no other symptoms or signs or laboratory findings to suggest Hodgkin's disease.

Although the authors' patient did not survive postoperatively, autopsy failed to reveal Hodgkin's disease anywhere except in the operative specimen. The authors' data suggest that a radiographic diagnosis of extensive carcinoma of the stomach, in the absence of metastases, should not preclude attempt at operative treatment, since the diagnosis may be wrong and apparently removal of a stomach involved by primary Hodgkin's disease may result in cure.

S E

**PREGNANCY AND HODGKIN'S DISEASE—WITH A REPORT OF THREE CASES.** *S. C. Kardon.* From the Tumor Clinic and the Gynecological Department, Boston Dispensary, New England Medical Center, Boston, Mass. *Am J Obst & Gynec* 57: 282-293, 1949.

From an analysis of the literature relative to pregnancy in the course of Hodgkin's disease, 3 cases of which are described in detail by the author, it is concluded that this disease has no demonstrable influence on ovulation, fertility, or the obstetric aspects of gestation, parturition, or the puerperium. The disease was transmitted from the mother to the fetus across the placenta in 9 per cent of reported cases. No evidence was obtained to indicate that x-ray therapy results in injury to the shielded fetus. Artificial interruption of pregnancy, on the basis of coexisting Hodgkin's disease, appears therefore to be unwarranted.

C P E.

**CHEMOTHERAPY OF MALIGNANT DISEASE.** *A. Gellhorn and L. O. Jones.* From the Departments of Cancer Research and Medicine, College of Physicians and Surgeons, Columbia University, New York, N. Y. *Am J Med* 6: 188-231, 1949.

The chemotherapeutic value of microbial products, antitumor cytotoxic serum, folic acid conjugates and antagonists, stilbamidine, urethane, androgens, estrogens, and nitrogen mus-

rards is comprehensively and critically reviewed from the standpoint of investigative efforts and rationale leading to their clinical use, mechanism of action and clinical application and limitations, 186 references are included

The authors have drawn the following conclusions (1) androgen therapy is warranted in prostatic malignancy no longer localized, (2) urethane is of value in chronic myelogenous and lymphatic leukemia when x ray therapy cannot be used, (3) nitrogen mustards are useful adjuncts to radiotherapy in malignant lymphomas (4) after conventional therapy has failed, androgens in carcinoma of the breast with skeletal metastases and stilbamidine in multiple myeloma with intractable pain should be tried (5) antitreticular cytotoxic serum and teropterin have no proven beneficial effect on malignant disease, (6) estrogen treatment of carcinoma of the breast with soft tissue metastases in postmenopausal women needs further investigation and (7) microbial products to date have not proved effective in the treatment of human neoplasms

The concept that our failure to produce cures may be due to the fact that certain cells in a neoplasm behave like bacteria which are capable of developing resistant strains to chemotherapeutic agents, is interesting. From that point of view one wonders if the simultaneous administration of several potent chemotherapeutic agents early might not offer even greater therapeutic benefits than alternate therapeutic courses over a longer period of time

H B M

CHEMOTHERAPY OF LYMPHOMA AND LEUKEMIA *W Dameshek* From Tufts College Medical School and the Pratt Diagnostic Hospital Boston Mass Bull New England M Center 11 49-62, 1949

This paper presents a concise historical review of chemotherapy in the proliferative disorders of the white cells and summarizes the author's experiences with nitrogen mustard (described in Blood 4 335 1949) and with certain folic acid antagonists, including aminopterin, a methopterin, amino-20-fol and a binopterin. Of 21 cases of acute or subacute leukemia who survived more than four days after initial therapy, remissions occurred in 9. The most pronounced drug toxicity and greatest efficacy were demonstrable in the patients receiving aminopterin which proved to be as effective and toxic when administered orally as when given parenterally. It was apparently necessary to produce definite toxic manifestations in order to achieve a remission. Therapeutic complications included ulceration of tongue and mucous membranes, nausea, upper abdominal discomfort and diarrhea. Vascular purpura and an enhanced bleeding tendency were also observed.

It was concluded that the folic acid antagonists possess, in varying degrees, the capacity to induce remissions in about one third of the cases of acute and subacute leukemia both in adults and in children and in both leukemic and leukopenic forms. Clinical hematologic and at least partial marrow remissions occurred most commonly in lymphocytic and least often in monocytic leukemia. Although by no means curative, these agents were therapeutically effective to a degree suggesting that with increasing knowledge of cellular enzyme systems and their inhibitors, great improvement in the treatment of leukemia may be anticipated and that the successful control of this disease may ultimately be achieved.

C. P. E.

EFFECTS OF FOLIC ACID ANTAGONISTS INOCULATED IN EMBRYONATED EGGS *P F Wagley and H R Morgan* From the Thorndike Memorial Laboratory Boston City Hospital Boston Mass Arch Path 44 441-450 1948

Since 1928 when Sabin first reported that fraction R of liver extract would influence the development of primitive erythroblasts of living chick blastoderms, contradictory results have been obtained by various investigators. Muller (1930) and Hays, Last and Koch (1942) obtained negative results. Reimer (1938) observed nonspecific degenerative changes in the liver. More recently, Riggio (1942) has observed that chick embryos incubated 32-33 hours responded to liver extract in three ways: viz. reversal of the ratio of erythroblasts and micromegakaryoblasts, reduction in percentage of histioid cells and an increase of mitoses in prophase. The present article by Wagley and Morgan is important because it shows that when some folic acid antagonists are injected into the yolk sac of chicks incubated for six to eight days, hemopoiesis is definitely influenced. Blood islets are decreased in size and number, nuclei exhibit pyknotic karyolysis and karyorrhexis. The larger doses of antagonists shortened the time for survival of the embryos. It was possible to protect against this effect by using relatively large doses of folic acid but not

possible with the dosage of liver extract and vitamin B<sub>12</sub> used. In this connection it should be noted that Rusznayk, Löwinger and Lajtha (1947) reported that folic acid acts directly on megaloblasts in tissue culture. Methyl 4 aminopteroylglutamic acid was not as potent as 4 amino-pteroylglutamic acid and N<sup>10</sup> methylpterotic acid had no effect in relatively large doses. Experiments like this should be encouraged and extended.

O P J

THE BLOOD CELLS AND THE HEMOPOIETIC AND OTHER ORGANS OF DOGS GIVEN INTRAVENOUS INJECTIONS OF 2-CHLOROETHYL VESICANTS. *J E Kinsler*. From the Anatomical Laboratory, University of Virginia, Charlottesville, Va. Arch. Path. 47: 378-399, 1949.

The present experiments reinvestigate on dogs experiments of a similar nature previously conducted on rats. Although the data are of a similar nature, better information concerning the daily changes in the blood picture were obtained. The material consisted of 17 dogs and the vesicants used were bis (2-chloroethyl) sulfide dissolved in thiodiglycol, the hydrochlorides of ethyl bis (2-chloroethyl) amine and tris (2-chloroethyl) amine dissolved in isotonic sodium chloride solution just before being injected into the saphenous vein. The results indicate that not only are vesicants rapidly acting specific poisons, but that they have a slower, more general intoxicating effect. Secondary pathologic changes occur in the organs which are believed to interfere with their proper functioning.

O P J

MULTIPLE MYELOMA. ITS CLINICAL AND LABORATORY DIAGNOSIS WITH EMPHASIS ON ELECTROPHORETIC ABNORMALITIES. *W S Adams, E L Alling and J S Lawrence*. From the Departments of Medicine and Radiology, University of Rochester School of Medicine and Dentistry, and the Medical Clinics of the Strong Memorial Hospital and the Rochester Municipal Hospital, Rochester, N. Y. Am. J. Med. 6: 141-161, 1949.

Sixty-one cases of multiple myeloma were analyzed and emphasis was placed on the most common and characteristic clinical and laboratory findings. A large section of this presentation is devoted to the electrophoretic study of the plasma protein abnormalities. These studies were considered of particular value in the differential diagnosis of this disease.

Of the 30 cases of multiple myeloma studied electrophoretically, 21 showed major abnormal patterns with tall, narrow peaks; 8, without such peaks, presented slight but significantly irregular pattern abnormalities; and 1 case of solitary myeloma of the autum with chronic infection showed patterns consistent only with infection.

The association of Bence Jones protein with multiple myeloma is discussed. It was observed that the incidence of Bence Jones proteinuria was low in the group with large abnormal peaks in their electrophoretic patterns but high in those patients with only small abnormalities. It is suggested that these small abnormal peaks were due to Bence Jones protein in the plasma and the Bence Jones proteinuria occurred in these patients because of the absence in the plasma of a protein of high molecular weight capable of forming complexes with Bence Jones protein.

Undoubtedly, electrophoretic studies will prove of great value in the future in our objective evaluation of the beneficial effects in this disease of the various chemotherapeutic agents.

H B M

## RADIATION EFFECTS

ABERRANT TISSUE DEVELOPMENTS IN RATS EXPOSED TO BETA RAYS. THE LATE EFFECTS OF P<sup>32</sup> BETA RAYS. *P S Hinshaw, R S Snider and E F Riley*. From the Clinton National Laboratory, Biology Division, Oak Ridge, Tenn. Radiology, 52: 401-415, 1949.

The exposure of rats to large single or repeated doses of beta rays from P<sup>32</sup> externally placed resulted in the appearance in the rats, some ten to twelve months later, of a large variety of tumors of the skin and subcutaneous connective tissue. Rats were exposed either to single doses of beta irradiation from plastic materials containing radioactive phosphorus or to repeated daily doses for a period of months. If the dose was sufficiently low, there were no effects of the irradiation. When single doses of 4,000 to 6,000 rep (roentgen equivalent physical) were given, typical changes occurred as follows. In one week,



acute skin erythema was followed by desquamation alopecia ulcers and in some rats death in four weeks Rats which survived showed healing of ulcers blindness, falling off of the ears alopecia telangiectasia of the skin and ultimately, neoplasms The neoplasms were of all varieties seen in the skin and connective tissue and were malignant Certain nonmalignant changes (production of extra claws soft tissue papillomata) also occurred

These changes were less marked at single doses of 8 000 rep suggesting an optimal range for their production They were much less marked in certain of the animals exposed to small daily doses over a period of months Tumors did not occur in control males and were much less frequent in control females

No conclusions are drawn which might relate to human usage of internal beta irradiation (i.e. in clinical application of  $P^{32}$  therapy) The range of dosage in the authors' experiments was extremely large (1 rep is the amount of ionizing radiation produced in one gram of tissue by 1 roentgen) and outside the clinical range The conclusion however that this type of irradiation resulted in a change in the destiny of certain cells is obviously tenable and of great interest in questions of growth

S. E.

## BONE MARROW

**NORMAL AND PATHOLOGIC PHYSIOLOGY OF THE BONE MARROW** *W. W. Zuelzer* From the Anemia Clinic of the Children's Hospital of Michigan and the Department of Pediatrics Wayne University College of Medicine Detroit Michigan *Am J Dis Child* 77 482-502, 1949

Several interesting and logical, although admittedly controversial, concepts are presented in this excellent discussion of normal bone marrow physiology and the functional disturbances associated with various disease states

Normal hemopoiesis involves three distinct functions (1) multiplication of precursor cells by mitosis, (2) maturation of cells and (3) release of mature elements Mitosis and maturation are considered as opposing although finely integrated tendencies in the development of the marrow cells While both processes normally occur in nearly all stages of cellular development mitosis predominates in younger cells and maturation in the more highly developed cells with a fairly even balance between the two in the intermediate stages In various pathologic states the functions of multiplication and maturation become dissociated Thus if mitotic function is lost in the more highly differentiated cells the number of immature cells increases markedly due to the depletion of the more mature cells and to the predominance of mitosis over maturation at primitive levels Maturation of such cells is therefore scanty slow and abnormal

Perhaps the most controversial point in the discussion is the concept that all immature red and white cells which appear in the peripheral blood in abnormal conditions (e.g. leukemia) come from extra medullary foci of hematopoiesis rather than from the bone marrow Several observations are cited to support this viewpoint In certain conditions in which extramedullary hematopoiesis can be demonstrated or assumed this is easily conceivable but there would appear to be noteworthy exceptions The answer must await a more thorough understanding of the mechanism of release of cellular elements from the bone marrow

H. W. B.

**STUDY OF FIXED TISSUE SECTIONS OF STERNAL BONE MARROW OBTAINED BY NEEDLE ASPIRATION III METASTATIC CARCINOMA IN STERNAL BONE MARROW** *A. S. Weissberger and R. W. Herold* From the Department of Medicine Lakeside Hospital and the School of Medicine Western Reserve University Cleveland Ohio *Am J M Sc* 217 263-268 1949

Of 50 selected patients with malignant tumors 7 were found to have metastatic lesions in the sternal bone marrow as demonstrated in tissue sections of particles obtained by needle biopsy Sternal marrow metastases were found only in the case of those tumors which have a tendency to metastasize to bone Metastatic lesions were found in the marrow in 2 patients after operations for carcinoma of the lung and breast respectively In one patient a metastatic lesion in the marrow was the only positive anterior roentgen evidence of carcinoma This would indicate that the procedure might be of benefit preoperatively in cases of carcinoma which are likely to metastasize to bone, and as a diagnostic tool in selected cases

G. E. C.

## ANEMIA

THE CLINICAL AND ROENTGEN MANIFESTATIONS OF ERYTHROBLASTOSIS FETALIS *M. Ritvo, I. A. Shaffer, and G. Kresnick* From the Departments of Radiology and Obstetrics Boston City Hospital, Boston Mass. *Am J Roentgenol* 61: 291-301 1949

Prepartum fetal roentenographic findings in erythroblastosis are described in 4 cases. These changes include increased bone density, zones of decreased density in the long bones at the cartilaginous junction, soft tissue edema, evidence of fetal death.

It would be desirable to have information on a much larger number of cases before any possible value of these changes in anticipating erythroblastosis can be evaluated.

C A F

FOLIC ACID IN COELIAC DISEASE. A STUDY OF ITS ADMINISTRATION IN TWENTY-TWO CASES *J. D. Hay* From the Royal Liverpool Children's Hospital and University of Liverpool, England. *Arch Dis Childhood* 23: 220-224 1948

It had previously been noted that although the macrocytic anemia associated with certain cases of coeliac disease responded favorably to pteroylglutamic acid, other types of anemia in such cases did not show any response to the material. The authors treated 22 children in whom they were able to establish a diagnosis of coeliac disease. None of these children apparently had fibrocystic disease of the pancreas. None of these cases had macrocytic anemia.

For a preliminary period of one to two months treatment consisted of a low fat high protein diet with added liver extract and vitamins. After this period pteroylglutamic acid was added to the regimen for a comparable period of time. The dose of folic acid was 20 or 10 mg. daily for one to two months. There was no particular change in the laboratory or clinical status of the patients on folic acid, and the author concluded that this material had no effect on coeliac disease in cases in which a macrocytic anemia (and megaloblastic marrow) were absent.

S E

CHRONIC HYPOPLASTIC ANAEMIA ARISING IN INFANCY *T. Robson and P. J. Sweeney* From the Royal Victoria and West Hants Hospital, London, England. *Arch Dis Childhood* 23: 294-296 1948

The subject of this report was pale at birth and showed at the time of initial examination at the age of 18 months pallor, slight hyponutrition, mental retardation and ptosis of one eyelid (which was present congenitally). The liver, spleen and lymph nodes were normal. The red cells numbered 1,230,000 per cu. mm., with a hemoglobin of 20 per cent (2.8 Gm.). The white cells and platelets were normal. Reticulocytes were virtually absent and a bone marrow aspiration showed a marked reduction of erythroid activity. A subsequent bone marrow biopsy showed absence of red cell precursors. Treatment was of no avail, except blood transfusions which were necessary and sufficient to maintain the child's blood at reasonable levels.

According to the authors there are very few reports of pure red cell anemia in the literature, perhaps 6 in children and 7 in adults. This report therefore is the seventh in a child. Of importance state the writers is possible spontaneous remission following repeated blood transfusion over a long period of time, hence continued treatment although without optimism is indicated.

S E.

SICKLE-CELL ANEMIA: ITS PATHOLOGICAL AND CLINICAL SIGNIFICANCE. *H. R. Pratt Thomas and P. A. Switzer* From the Departments of Pathology and Medicine, Medical College of the State of South Carolina, Charleston S. C. *South M J* 42: 376-384, 1949

Ten cases with necropsy findings are presented in which profound sickling of the red cells was the outstanding and often the only significant finding. None of the patients had active sickle cell disease. In several cases ischemic lesions were seen in the spleen, kidney or brain although thrombus formation was not demonstrable in the vessels supplying these areas.

The question is raised as to whether vascular obstruction is produced primarily by the masses of agglutinated sickle cells observed histologically or whether sickling and agglutination are merely secondary phenomena under conditions of stasis and anoxemia. Regardless of which is the initiating factor

these cases do emphasize the potential hazard of sickleemia in conditions producing lowered oxygen tension such as shock, anesthesia fever and congestive failure

H. W. B.

**RESPONSE OF LINGUAL MANIFESTATIONS OF PERNICIOUS ANEMIA TO PTEROYLGLUTAMIC ACID AND VITAMIN B<sub>12</sub>** J. F. Schieve and R. W. Rundles From the Department of Medicine Duke University School of Medicine Durham N. C. J. Lab. & Clin. Med. 34 439-447 1949

Two patients with pernicious anemia in relapse taking 30 and 50 mg. of pteroylglutamic acid daily developed acutely sore tongues with mucosal atrophy during the third month of therapy. The lingual mucosal atrophy of 5 patients with untreated pernicious anemia responded in five to seven days to injection of vitamin B<sub>12</sub>. The authors mention that in 6 of their patients treated with pteroylglutamic acid whose lingual responses were poor or who later relapsed, the blood levels remained below normal. Neurologic disease progressed in one and the red cell count fell significantly in another. They state in conclusion "The therapeutic limitations of pteroylglutamic acid in pernicious anemia relate to all manifestations of the disease — anemic, neurologic, and lingual—rather than to merely the neurologic."

G. E. C.

**BACTERIMETRIC STUDIES. III. BLOOD LEVEL STUDIES ON TEROPTEIN METABOLISM** G. Toennies and D. L. Gallant From the Lankenau Hospital Research Institute and the Institute for Cancer Research Philadelphia Pa. J. Lab. & Clin. Med. 34 501-508, 1949

Studies using *L. casei* and *Str. faecalis* of the fate of pteroylglutamic acid in human subjects indicated that two hours after intramuscular injection approximately two-thirds of the dose was present in the circulation. Of this total about two-thirds was present as the mono- and one third as the triglutamate. Subsequently the concentration of the monoglutamate declined more rapidly than the triglutamate. Different individuals showed considerable variations from the average metabolic pattern.

G. E. C.

**MEGALOBlastic ANAEMIA IN AN INFANT** J. H. Hutcheson and P. MacArthur From the Department of Child Health University of Glasgow and the Royal Hospital for Sick Children Glasgow, Scotland Lancet 1 916-917 1949

A case is reported of a girl who at 12 months developed severe anemia following enteritis. She responded to treatment with liver and iron but relapsed after a further attack of diarrhea and vomiting and was readmitted to hospital at 17 months. On investigation the anemia proved to be megaloblastic and responded to folic acid treatment.

S. C.

**THE MARCHIAFAVA MICHELI SYNDROME (PAROXYSMAL NOCTURNAL HAEMOGLOBINURIA)** J. Marks From the Department of Medicine University of Cambridge and the Bland-Sutton Institute of Pathology Middlesex Hospital London England Quart. J. Med. 18 105-121 1949

The authors present a thorough review of the literature and report 3 additional cases of Marchiafava-Micheli syndrome, bringing the total reported at the time of writing to 76. The review emphasizes the abnormality in the erythrocytes which undergo lysis by a thermolabile component of normal serum. Hemolysis is inhibited by sodium citrate, potassium oxalate, potassium cyanide, and heparin. The possible efficacy of pilocarpine nitrate in treatment is mentioned, a favorable influence on hemolysis apparently being observed in one case. The drug in this instance had to be discontinued because of undesirable side effects. Splenectomy is ineffective and is accompanied by a 40 per cent operative mortality in the cases where it has been attempted. Since hemolytic anemia without hemoglobinuria may dominate the clinical picture, the necessity of performing the specific serologic tests for this disease in any obscure hemolytic anemia is apparent.

W. N. V.

**ON PERNICIOUS ANEMIA IN MYXEDEMA** H. Esser and F. E. Schmengler Dtsch. Arch. Klin. Med. 193 431 1948

Three forms of anemia are seen in myxedema: (1) The uncomplicated myxedemic anemia (endocrine type); (2) the hypochromic anemia (endocrine type plus sideropenia); (3) (very rare) the hyperchromic form similar to pernicious anemia.

The author gives a description of a typical case of the third form. The following characteristics differentiate the myxedemic pernicious anemia from the typical pernicious anemia: (1) Unusually high color index (not treated 1.9), (2) inhibition of hemolysis, (3) inhibition of bone marrow activity, (4) course free from fever and relative bradycardia despite severe anemia, (5) normal or slightly reduced basal metabolism.

The endocrine disturbance prepares the way for pernicious anemia.

C M

INVESTIGATIONS OF THE RED CELL PICTURE UNDER THE CHRONIC INFLUENCE OF BENZENE AND ITS DERIVATIVES WITH SPECIAL REGARD TO THE DIAETER OF ERYTHROCYTE. *Karl Humperdinck and Alfons Abler* (Arbeitsmedizin. Institut Stuttgart/Tübingen.) *Aerzt. Forsch.* No. 5, 117-120, March 10, 1949.

Mixed solutions of benzene and its derivatives as used in industry damage the leukopoietic system and cause a moderate anemia of the hyperchromic type. This anemia is characterized by a slight increase of the average diameter of the red cells. Investigations based on 20 cases illustrate the diagnostic importance of this hyperchromic anemia as an early symptom.

C M

CELLS OF THE MEGAKARYOCYTE SERIES IN PERNICIOUS ANEMIA. IN PARTICULAR THE EFFECT OF SPECIFIC THERAPY. *R. D. Epstein* From the Thorndike Memorial Laboratory, Boston City Hospital, Boston. *Mass. Am. J. Path.* 35, 239-251, 1949.

In the past the many studies of pernicious anemia marrow during relapse have reported a decrease in megakaryocytes in general, and alterations in nuclear segmentation and lack of azurophilic granules in some instances. In the present study, marrow was aspirated from 5 patients before therapy was begun and again after the reticulocyte response had occurred. The number of megakaryocytes was estimated in terms of a million nucleated cells within about 20 oil immersion fields. Two general classes of megakaryocytes were recognized, viz. the mononuclear group and the group containing multiple nuclei in a single cytoplasmic mass. The latter are referred to as polykaryocytes of which there may be young, intermediate and mature forms. During relapse the percentage of polykaryocytes was high and that of the mononuclear megakaryocytes low. After remissions were induced by liver extract therapy the ratios were reversed. Whole blood and red cell concentrate transfusions did not produce this reversal. In other words the megakaryocytic system in pernicious anemia during relapse needs something other than an increase in the oxygen-carrying capacity of blood.

O P J

CHRONIC FAMILIAL METHEMOGLOBINEMIA AND A NEW MODIFICATION OF METHEMOGLOBIN. *H. Herlein and G. Weber* (Inn. Abt. der Stadt. Krankenhäuser Wuppertal-Elberfeld.) *Dtsch. Med. Wschr.* 1948, 476-478.

A family with methemoglobinemia is described in whose case, contrary to former observations, heredity was not recessive but dominant. Furthermore the pigment was not the usual methemoglobin but a new modification with a maximum absorption band at  $600\mu$ . The author believes that this variation is due to a specific change in the globin component of the hemoglobin molecule.

C M

# NEWS AND VIEWS

## THE INTERNATIONAL SOCIETY OF HEMATOLOGY

The following News Letter (May 1949, Vol I, No 1) has been received from The International Society of Hematology, Office of the Secretary General, Western Hemisphere

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### The Second Congress Plans to Date

The Society will hold its Second Congress in Cambridge, England, August 21-26, 1950 Reports of plans and progress from President Sir Lionel Whitby, and Secretary-Treasurer of the 1950 Congress, Dr Martin Hynes, are indicative of an excellent meeting

Living accommodations are being planned at the University of Cambridge and in Cambridge The living quarters are being arranged to accommodate members and their families The Society will be glad to receive visitors who desire to attend

the Congress Applications for living quarters will be sent to those desiring such assistance at a later date

### LETTER TO THE EDITOR

The following letter has been received from Dr A Piney of London, England (For reference, see "Revised Nomenclature Pro and Con, *Blood*, June 1949, pp 776-782, for the original article, source of the discussion, see "Condensation of the First Two Reports of the Committee for Clarification of the Nomenclature of Cells and Diseases of the Blood and Blood-forming Organs, *Blood*, January 1949, pp 89-96)

TO THE EDITOR

I appreciate that you may not be able to publish an extensive correspondence on the proposed standardization of hematological nomenclature but I hope that you may be able to find space for a few brief remarks

(i) Those who, like myself can look back more than a quarter of a century will have noticed that there is far greater international agreement about the names of blood-cells than there used to be. This has come about by evolution in fact, by what Dr Wintrobe (p 777) advocates viz advancing knowledge rather than in too much concern about names

(ii) Dr O P Jones points out (p 777) that hematology is international in its scope and as a consequence, its terminology is not the property of any one country but in contradiction to this Drs Osgood Kracke and Heck assert "The problem seems sufficiently difficult to settle in *one* language at a time

Is not the only valid excuse for a terminology based on the classical languages that it can be international?

The proposals of Dr Osgood and his colleagues are such as to be quite useless to those who do not speak English. Rubriblast is a nasty etymological bastard but it is not so parochial a term that a Frenchman or a German could not understand it, but pernicious anemia type is incomprehensible to him

(iii) Even you Sir inadvertently demonstrate that evolution is a more satisfactory method of progress than is dictation. You say (p 782) "Certainly, consistency is always something to be applauded so why not use for leukemia the terms myelocytic lymphocytic and monocytic rather than myelogenous lymphatic and monocytic?" The answer to your question is of course that not all myelogenous leukemias are myelocytic or all lymphatic ones lymphocytic

A foolish consistency is the hobgoblin of little minds. Speak what you think to-day in words as hard as cannon balls and to-morrow speak what to-morrow thinks in hard words again.<sup>1</sup>

(iv) A Committee appointed by the Minister of Public Health in France has produced a nomenclature which is relatively simple and which does not represent a break with the history of hematology. Even so Chevallier<sup>2</sup> has published an alternative series of suggestions while attacking the quasi-official one sponsored by the Ministry

Are we not Sir in danger of being precipitated into the same sort of sterile arguments that filled hundreds of pages in the early volumes of the *Folia Haematologica*? And should we not do better to continue as in the past by defining our terms whenever necessary until some time in the future a labile but inherently stable terminology comes to birth, thus allowing us to avoid the risks of malformation that are inherent in all premature births?

ALFRED PINEY M.D.

<sup>1</sup> Emerson Ralph Waldo Essays 11 Self Reliance

<sup>2</sup> Chevallier P Rapport pour servir à la discussion sur la nomenclature des cellules du sang Sang 330-348, 1949

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# BOOK REVIEW

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*Bone Marrow Biopsy Hematology in the Light of Sternal Puncture* S J LEITNER (Switzerland) English Translation Revised and Edited by C J C BRITTON AND E NEUMARK of London Grune & Stratton New York 1949 \$8.50 433 pages

If one were to single out the one technique which has done most to advance the cause of hematology in the last two decades marrow aspiration would probably be well in the foreground. Despite this there has been a real dearth of good texts dealing with marrow biopsies. Britton and Neumark have therefore done a fine service for the English speaking medical profession in translating and revising Leitner's monograph. The result is a comprehensive 433 page work replete with hundreds of references to the current literature, 194 text figures and 6 color plates. The techniques and methods involved in sternal puncture are well described. Unfortunately there is no description of the sternal trephine biopsy nor of puncture aspirations at other sites, i.e. spinous process of the vertebrae, the iliac crest, etc.

The various marrow cells are carefully described. An unusual feature is a description of such matters as mitoses, maturation curves, karyokinetic curves and abnormal cell division.

Excellent descriptions are given of the marrow in pernicious anemia, idiopathic hypochromic anemia and in such miscellaneous disturbances as the anemia of hemorrhage, infection and nephritis. The descriptions of the marrow in polycythemia vera and aplastic anemia are by no means as comprehensive, however, and below the standards set for thrombocytopenic purpura and tumors.

Descriptions of individual cells are excellently done. The color plates show excellent color reproduction. On the other hand, some of the photomicrographs of single cells leave somewhat to be desired and could have been arranged with more uniformity. The bibliography is exhaustive and references to the literature are not only well chosen but complete.

This book is the first comprehensive text on marrow aspirations and as such should be required reading for all those having any interest in clinical and investigative hematology.

WILLIAM DANESHK

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## *Contributors to this Issue*

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C THOMAS DEBERARDINIS, M D

Associate in Medicine Jefferson Medical College  
Philadelphia Penna

MATTHEW BLOCK, M D , Ph D

Department of Medicine University of Chicago  
Chicago Ill , Senior Research Fellow (Cancer)  
United States Public Health Service

VIRGINIA R HAWKINS, R N

Research Assistant in Internal Medicine College  
of Medicine University of Cincinnati Cincinnati  
Ohio

F HERNANDEZ-MORALES, M D

School of Tropical Medicine Department of  
Medicine San Juan Puerto Rico

GEORGE M HIGGINS, Ph D

Division of Experimental Medicine Mayo Foun-  
dation, Rochester Minn , Professor of Experi-  
mental Medicine, the Graduate School Univer-  
sity of Minnesota Minneapolis Minn

F HOMBURGER, M D

Associate, Sloan Kettering Institute for Cancer  
Research New York City Present address Tufts  
College Medical School Department of Surgery  
Cancer Research and Cancer Control Unit Boston  
Mass

JOSEPH M LUBITZ, M D

Department of Pathology Veterans Administra-  
tion Center Wood Wisc Marquette University  
School of Medicine Milwaukee, Wisc

JOHN F MUELLER, M D

Fellow in Internal Medicine, College of Medicine  
University of Cincinnati Assistant Clinician  
Medical Clinic Cincinnati General Hospital Cin-  
cinnati Ohio

ENRIQUE PEREZ, M.D

School of Tropical Medicine, Department of  
Medicine San Juan Puerto Rico

M L PETERMANN, Ph D

Associate Sloan Kettering Institute for Cancer  
Research New York City

HOBART A REIMANN, M D

Professor of Medicine, Jefferson Medical College  
Philadelphia Penna

TOM D SPIES, M D

Northwestern University Department of Nutrition  
and Metabolism Hillman Hospital Birmingham,  
Ala.

L A STERNBERGER, M D

Department of Pathological Physiology, Hebrew  
University and the Rothschild Hadassah Univer-  
sity Hospital Jerusalem Present address Sloan  
Kettering Institute for Cancer Research Memorial  
Cancer Center New York City

RAMON M SUAREZ, M D

Head of Department of Clinical Medicine School  
of Tropical Medicine San Juan Puerto Rico

RICHARD W VILTER, M D

Associate Professor of Medicine and Assistant  
Dean College of Medicine University of Cin-  
cinnati Attending Physician Department of  
Internal Medicine and Chief Clinician Medical  
Out Patient Dispensary Cincinnati General Hos-  
pital Cincinnati Ohio

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# BLOOD

## *The Journal of Hematology*

VOL IV, NO 10

OCTOBER, 1949

### STUDIES ON HYPOPROTEINEMIA II FAMILIAL IDIOPATHIC DYSPROTEINEMIA

By F HOMBURGER, M D, AND M L PETERMANN, PH D

THE PRESENT paper describes a new familial syndrome characterized by subtle disturbances of the qualitative relationship of the components of the blood plasma proteins and by a number of clinical disturbances

The patients comprising this study provided a unique opportunity to investigate mechanisms of the homeostasis of plasma proteins in individuals not afflicted with any of the common pathologic causes affecting the metabolism of plasma proteins. Studies were thus possible on the homeostasis of plasma proteins, i e, the factors which maintain uniformity or stability at the normal levels

In view of the many factors which tend to unbalance the equilibrium of plasma proteins this homeostasis is remarkably effective, yet little is known regarding its mechanisms. The known facts may be summarized as follows. Deviations from the norm (euproteinemia) may result in hypoproteinemia, hyperproteinemia, dysproteinemia and paraproteinemia—the last two terms meaning a disproportion between components usually occurring in blood plasma (dysproteinemia) and the presence of proteins not usually found in blood (paraproteinemia). A combination of any of these disorders with each of the others is of course possible. Thus, in multiple myeloma hyper-, para-, and dysproteinemia may coexist,<sup>17</sup> and gastric cancer<sup>18</sup> may cause hypo- and dysproteinemia (hypoalbuminemia). A similar picture is found in nephrosis.<sup>6</sup> In Addison's disease,<sup>19</sup> one may find dysproteinemia with normal total protein levels or with hyperproteinemia, and in dehydration hyperproteinemia may exist without disturbance of the proportional relationship of plasma components. With the exception of dehydration and of the production of Bence Jones protein in multiple myeloma, the mechanisms causing these disturbances are poorly understood. Even in the relatively simple case of hypoproteinemia the mechanisms are complex. It may occur because the circulating blood is inadequately supplied with protein or because too much of it escapes. Insufficient protein enters the blood stream in malnutrition or intestinal malabsorption or in defective synthesis of proteins (liver disease,<sup>20</sup> Cushing's syndrome<sup>4</sup>). Protein

From the Departments of Clinical Investigation and Protein Chemistry, the Sloan Kettering Institute for Cancer Research, Memorial Cancer Center, New York. This study has been aided by grants from the Teagle Fellowship Foundation, the National Cancer Institute, the Finney Howell Research Foundation, and the James Foundation of New York, Inc. With the technical assistance of Lee Burnett, Iris Forbes, Anita Ursloff, and Charlotte Pann. The electrophoretic analyses were performed by F P Hogness and Barbara Brodsky Gottlieb.

is lost in excess from the blood stream in nephrosis,<sup>5</sup> in altered vascular permeability, as in burns,<sup>6</sup> or with excessive catabolism of proteins, as in hyperthyroidism, uncontrolled diabetes mellitus<sup>8</sup> and pyrexia

A more complex type of hypoproteinemia which persists in the presence of tissue protein repletion occurs in patients with gastric cancer,<sup>9</sup> in chronic tuberculosis,<sup>10</sup> and in certain types of kidney disease.<sup>11</sup> In another group of cases the hypoproteinemia is unexplained and is therefore designated idiopathic hypoproteinemia.<sup>12-16</sup>

It appears from the preceding discussion that a theory of simple depletion alone cannot explain all of these phenomena.<sup>20-21</sup> Although many aspects are still but little understood, an approach to some of them is possible in favorable circumstances.

In the patients studied by us, a number of physiologic factors governing the homeostasis of single protein components have been observed. There were none of the usual systemic disorders leading to changes of the protein pattern and, except for the dysproteinemia and idiopathic hypoproteinemia in some cases, the individuals were healthy.

A detailed description of the clinical syndrome is necessary for the proper interpretation of the experiments to be described.

## PART I THE CHARACTERISTICS OF THE SYNDROME AND ITS FAMILIAL ASPECTS

After the clinical studies described below, it became evident that the subjects studied presented a new syndrome, for which the name familial idiopathic dysproteinemia is proposed. The syndrome is characterized by the familial occurrence of edema of the legs, with ulcers in the males and functional vascular changes in the females, by dysproteinemia of variable types and sometimes discernable only by electrophoresis, by a number of congenital malformations and by a high incidence of stillbirths. No etiologic factor was found.

### *Methods of Study*

A complete history was taken in each available member of the family and checked against that given by every other member. A complete physical examination was made of each available member. Whenever possible routine studies of renal, hepatic,\* cardiovascular (including oscillometric studies by means of the Collins oscillometer), gastro intestinal and adrenal<sup>22-27</sup> function were carried out. In the case of some patients special tests were employed. These included liver biopsy through an abdominal incision in one case (Case 8) and muscle biopsies for study of blood vessels in two cases (Cases 5 and 8) as well as measurements of the renal clearance of glucose, para aminohippuric acid and mannitol.<sup>28-30</sup> Glucose tolerance tests<sup>31</sup> and nitrogen, phosphorus and potassium balance studies<sup>32</sup> were performed on the metabolic ward.

The total plasma protein concentration was measured by Kjeldahl analysis and corrected for non-protein nitrogen. Plasma volumes were estimated by the Evans blue method.<sup>33</sup> The volume of the extracellular fluid space was estimated by the use of thiocyanate.<sup>34</sup> The electrophoretic technique used in this study has been described elsewhere.<sup>35</sup> Unless otherwise specified a veronal-citrate buffer at pH 8.6 and ionic strength 0.10 was used. Electrophoresis was performed on samples of plasma obtained from the patients

\* In the liver function tests the following methods were employed: measurement of hippuric acid excretion,<sup>36</sup> retention of bromsulfalein,<sup>37</sup> cephalin flocculation test,<sup>38</sup> and thymol turbidity test.<sup>39</sup>

TABLE 1—Nitrogen Phosphorus and Potassium Balance Study during 18 Days See also Fig. 3

Average N intake per day	28.0 Gm
Average N output* per day	24.5 Gm
N Utilized per day	3.5 Gm
K Utilized per day	6.2 mM.
P Utilized per day	145 mg

\* Stool 0.85—7 Gm per day

TABLE 2—Case 8 Tabulation of Total Protein Concentration Albumin Concentration and Protein Intake from December 1945 to October 1947

Plasma means the 975 cc. of plasma injected at that time containing 6.15 Gm of protein per 100 ml. Notice that the actual (electrophoretically measured) albumin is considerably lower than that measured by the Howe method. The Howe albumin roughly corresponds to the sum of the electrophoretic albumin plus the globulins.

Date	Albumin Gm 100 ml		Total Plasma Protein	Protein Intake Gm/day
	Electrophoresis	Howe		
12/-/45	Rockefeller Inst.	3.57	5.28	
3/-/46		3.53	4.06	
6/-		3.25	4.9-	
11/11				
11/19	2.25		5.08	↑
11/20		3.23	4.31	↓
11/25		3.19	4.52	
12/4	1.95 (+α <sub>1</sub> + α = 3.24)	3.18	4.58	↑
12/13			4.65*	↓
1/23/47	2.75		5.31	75
Plasma 1/27			6.15	↓
1/27	2.96		5.49	
1/28			6.01	
1/30	3.05		5.83	
2/1	2.79		5.27	↑
2/5	2.68		5.13	75
2/21			4.95	↓
3/8			4.70	
Albumin				
5/1			5.16†	
Plasmaphoresis				
5/8			3.60	
5/27			4.4-	
Hepatitis				↑
6/13	0.93 (+α <sub>1</sub> + α = 2.08)	1.81	3.60	110
6/18		3.48	4.73	
6/21		3.38	5.13	
6/24		3.37	4.59	
7/22	2.66 (+α <sub>1</sub> + α = 3.91)	3.85	5.1	
10/8	2.08 (+α <sub>1</sub> + α = 3.04)		4.04	
10/10	2.29 (+α <sub>1</sub> + α = 3.14)		4.58	
10/15	~3- (+α <sub>1</sub> + α <sub>2</sub> = 3.40)		4.70	↓

\* Plasma vol 3000 ml

† Plasma vol ~360 ml

in the postabsorptive state. Since values for the various protein components given as percentages may be misleading when the total protein concentration is low, the concentration of each component is given in grains per 100 ml. of plasma. The amino acids of plasma and urine were studied by chromatograms on paper<sup>26</sup> and no abnormalities were found.

The composite family history is indicated in the genealogic table in figure 1. The strong physical resemblance between some of the members of the family is evidenced in figure 2.

The mother, now 70 years old and a cardiac patient, had suffered from ankle edema since the age of 50. There were consistently large families on her side as well as on her husband's in both their generation and the preceding one. In contrast to that, she had only 6 living offspring out of 11 pregnancies. The still-born fetuses were described as edematous about 7 months old and uremic poisoning was given as the presumed cause of death. A stillbirth also occurred in the subsequent generation presumably in the third month of pregnancy. No information was obtained regarding this fetus.

The father's family history reveals 3 surviving brothers: one with long-standing edema of the legs, presumably due to varicose veins, and 2 with prominent floating ribs. The father had edema of the legs,

TABLE 3—Liver Function Tests in Cases 8, 5 and 7

Case no	Date	BSP	TT	C.F.	H.A.	PT
Normal		0	<17	0-1+	>1 Gm.	
8	12/45	30% (NYH)				88.75
	12/45	1% (R. I.)				
	11/46	0	60	0-1+	1.075	
	3/47	Negative	Liver biopsy			
	6/13	Hepatitis	320	4+		
	6/18		345	4+		
	6/25			3+		
	7/3			3+		
	7/22		105	3+		
	10/18		40	0		
	11/47	0	60	0	1.075	
5	12/46	4%	15	0	1.4038	
7	11/46	0	60	0-1+	1.206	

BSP—Bromsulfalein excretion test.

TT—Thymol turbidity test.

C.F.—Cephalin flocculation.

H.A.—Hippuric acid excretion test.

PT—Prothrombin time.

NYH—New York Hospital.

R.I.—Rockefeller Institute.

at times severe enough to be incapacitating, he underwent a number of operations for varicose veins. He died of dropsy at the age of 52, following a cholecystectomy. He had a double vortex pilorum, a malformation recurring in several of his offspring (V in fig. 1).

Eighteen members of the family are included in the present study. Eleven of these were examined at home and blood taken for electrophoresis (Cases II H, II M, 3, 4, 6, 10, 11, 12, 14, 15). Five were hospitalized (Cases 1, 5, 7, 8, and 9) for studies lasting from 1 day (Case 1) to 1 year (Case 8). Two were not seen (Cases II N and 2), the histories being obtained from relatives.

In 9 of these subjects ankle edema was present. It is of interest to note that none of the patients who were prepubertal or at puberty (Cases 10, 11, 12, 14, 15) had ankle edema, even though dysproteinemia existed in 3 of them (Cases 11, 14, and 15). The onset of edema in all of those affected had always been after puberty. Some form of dysproteinemia was found in all patients with edema. Of 7 adult males, 4 had ulcers of the legs or a history thereof.

The following physical signs were found. All adult males in generation III (Cases 3, 5, and 6) had ulcers of the legs at some time in their adult life. All adult females in generation III (Cases 4, 7, and 8) had low oscillometric indices in the upper extremities. Protruding floating ribs were seen in 11 subjects (Cases

II L, II M 3 4 5 7 8 10 12 14 15) Double vortices pilorum existed in 4 individuals 3 males and one female (Cases 2 5 9 and 15) scattered through 3 generations

#### Laboratory Studies (Tables 5 and 8)

Laboratory studies revealed the following Hypoproteinemia existed in 4 cases (Cases 5, 8, 9 and 14), ranging from 4.7 Gm per 100 ml to 6.0 Gm per 100 ml (The normal range in our 13 controls was from 6.1 Gm per 100 ml to 7.9 Gm per 100 ml) Dysproteinemia was found in 10 cases (Cases 5, 6, 7, 8, 9, 11, 14, 15, II L and II M) The changes found in the mother (Case 1) and in one of her daughters (Case 7) may be insignificant, and in Case 6 the marked abnormalities reverted to normal after 6 months In all the others, dysproteinemia was present beyond doubt A single component was altered in 7 instances ( $\alpha_2$ , in Cases II L, II M, 7 and possibly Case 5, albumin in Case 9 and possibly Case 1, and  $\gamma$ -globulin in Cases 11 and 14) \* Two plasma components or more were altered in 3 cases (Case 5, albumin,  $\alpha_2$ , and  $\gamma$ -globulin), Case 6 showed  $\alpha_2$  and  $\beta$  markedly changed on one occasion on repeated analyses in two different buffers Six months later, no abnormalities were uncovered by electrophoresis Case 8 repeatedly had a low albumin and exceedingly low  $\gamma$ -globulin values In Case 14 there was a low  $\gamma$ -globulin and a low albumin) \*

TABLE 4 —Kidney Function Tests in Case 5 and Case 8

	Mannitol (glomerular filtration) ml/min	PAH (renal plasma flow) ml/min	Glucose Tm mgm/min.
Normal Range	90-120	500-700	250-350
Case 5	107	645	322
Case 8	117	540	276

This represents a wide variety of alterations in the concentration of individual components of plasma protein with only one combination of changes (albumin and  $\gamma$ -globulin) occurring in more than one patient

Routine clinical laboratory findings were normal in all subjects studied Blood chemical examinations other than plasma protein levels (vide supra) were within normal limits There was a tendency to hypochlorhydria in the 3 subjects studied (Cases 5, 7 and 8) and no response to histamine in one case (Case 5) but proteolytic enzymes were present in the gastric juice of all patients The hematologic examination gave a normal picture, there was a tendency to low white counts but none fell below the normal range

Urine examinations were consistently normal and no proteinuria could be demonstrated by any of a number of methods

The renal clearance studies (table 4) and the adrenal function tests gave normal responses All liver function tests (table 3) gave negative results excepting in Case 8, in which liver functions were disturbed in the course of a severe homologous serum hepatitis This was followed by a return to normal function as measured by

\* Interpretation of the electrophoretic data in Cases 14 and 15 was difficult because of cloudiness caused by a meal being taken before venipuncture The patterns were fairly normal for children of this age <sup>27</sup> except for the changes noted

the tests. In Case II L, there was a history of alcoholism and clinically liver disease could be suspected but no liver function tests could be carried out.

Muscle biopsies showed normal blood vessels and lymphatics (fig 4) and the special stains and numerous sections studied on the liver biopsy material taken from Case 8 failed to reveal any morphologic change (fig 4).

TABLE 5—*Tabulation of Plasma Protein Components as Determined by Electrophoresis. The values falling outside our normal range are italicized. See also Fig. 3.*

Subject		Total Protein	Plasma Proteins in Grams/100 ml.					
			Albumin	$\alpha_1$	$\alpha_2$	$\beta$	$\phi$	$\gamma$
Normal Av		6.90	3.81	0.42	0.61	0.87	0.41	0.78
Normal Range		6.10-7.85	3.42-4.39	0.28-0.54	0.57-0.81	0.63-1.09	0.31-0.59	0.61-0.99
Std. Deviation		0.508	0.292	0.072	0.062	0.137	0.076	0.103
Case No	Date							
I	4/16/47	6.49	3.82*	0.51	0.64	1.03	0.58	0.72
I	4/9/48	7.18	3.64	0.44	0.63	1.12	0.60	0.76
II H	4/9/48	7.02	3.88	0.40	0.60	1.00	0.48†	0.86
II L	4/9/48	7.60	3.89	0.50	0.96	1.02	0.54	0.70
II M	4/9/48	6.91	3.48	0.44	0.89	0.96	0.51	0.63
3	4/9/48	6.36	3.67	0.46	0.65	0.74	0.30‡	0.55
4	4/9/48	7.24	3.79	0.45	0.64	1.01	0.54	0.81
5	1/2/47	5.28	3.05	0.49	0.36	0.77	0.31	0.39
6a	4/18/47	6.72	3.69	0.47	1.09	0.42	0.42	0.63
6b†	4/18/47	6.72	3.62	0.45	1.01	0.52	0.50	0.63
6c	4/9/48	7.31	4.04	0.48	0.77	1.01	0.34	0.67
7	11/22/46	6.24	3.59	0.46	0.49	0.77	0.34	0.59
8a	11/11/46	5.08	2.25	0.58	0.82	0.83	0.45	0.16
8b	12/4/46	4.65	1.95	0.49	0.80	0.79	0.45	0.16
8c¶§	1/23/47	5.31	2.90	0.42	0.55	1.27	—	0.21
8d¶¶	1/27/47	5.31	2.71	0.64	0.71	0.99	—	0.27
8c	4/9/48	4.92	2.60	0.41	0.76	0.86	—	0.19
9	4/9/48	5.99	3.00	0.41	0.76	0.72	0.38	0.72
10	4/9/48	6.74	4.12	0.38	0.66	0.64	0.23†	0.70
11	4/9/48	6.20	3.60	0.43	0.58	0.76	0.37	0.41
12	4/9/48	7.51	4.20	0.44	0.74	0.90	0.45	0.78

\* Values outside the normal range are italicized.

† Specimen partially clotted.

‡ Veronal buffer ionic strength 0.10, pH = 8.6.

¶ Serum.

§ Phosphate chloride buffer ionic strength 0.2, pH = 7.7.

¶ Veronal-citrate buffer with 0.2 M NaCl added pH = 8.2.

## PART II STUDIES ON THE NATURE OF THE DEFECTS OF PROTEIN HOMEOSTASIS IN FAMILIAL IDIOPATHIC DYSPROTEINEMIA

The following studies were made on the nature of the alterations of the plasma proteins.

1. A nitrogen balance study was made in Case 8. This patient was maintained in positive nitrogen balance for a considerable length of time, during this period her total circulating plasma protein was measured repeatedly.

2 In the same patient, the rate at which the increased albumin concentration returned to normal following the intravenous injection of human albumin was determined by measuring plasma volume and albumin concentration<sup>38 39</sup> before and after the injection of human serum albumin

3 In the same case, the rate at which  $\gamma$ -globulin concentration returned to normal following the injection of plasma containing a normal amount of  $\gamma$ -globulin was determined This was possible because the initial concentration of  $\gamma$ -globulin in the plasma was very low Nine hundred and seventy-five ml of pooled plasma were injected and the  $\gamma$ -globulin concentration before and after the plasma infusion was followed by electrophoretic studies and also by immunologic methods to be reported later<sup>40</sup> The results of the electrophoretic procedures were subjected to certain calculations before evaluation \*

TABLE 6—The Disappearance of Injected Plasma Protein Components in Case 8 with Special Reference to  $\gamma$  globulin (see text) Plasma protein concentration in Grams/100 cc

Sample	Total Protein	Albumin	$\alpha_1$	$\alpha_2$	$\beta$	$\phi$	$\gamma_1$	$\gamma_2$	$\gamma_2$ corr
1/27/47 pre inj	5 31	2 75	0 59	0 82	0 90	—†	0 06	0 19	n 28
1/27/47 2 hrs post-inj	5 49	2 96	0 52	0 76	0 81	—†	0 10	0 35	0 51
1/28/47	6 01								
1/30/47	5 83	3 05	0 56	0 89	0 92	—†	n 09	n 32	n 47
2/1/47	5 27	2 79	0 46	0 82	0 85	—†	n 08	0 28	0 41
2/5/47	5 13	2 68	0 52	0 76	0 81	—†	0 09	0 26	0 38
Plasma Pool	6 15	3 32	0 45	0 60	0 70	0 37	—	n 71	1 04

\*  $\gamma_1$  is the globulin component of serum which has the mobility of fibrinogen. (Biophysical studies on blood plasma proteins IV Separation and purification of a new globulin from normal human plasma Deutsch H F Alberty R. A, and Gosting L. J. J Biol. Chem., 165 31-25 1946)  
† Serum

4 In the same patient, the effect on the plasma protein concentration of the acute withdrawal of 500 ml of blood and reinjection of the cells into the donor was studied (protein subtraction test<sup>41</sup>) After a base-line sample had been taken, 500 ml of blood were removed, centrifuged in a closed system and the red cells separated from the plasma The cells were suspended in 10 per cent dextrose solution to make a total volume of 500 ml and immediately reinjected The plasma protein concentration was then followed<sup>42 44</sup>

\* From the data of Perlmann and Kaufman<sup>41</sup> and of Armstrong Budka and Morrison<sup>42</sup> it may be calculated that the  $\gamma$  globulin values obtained under the conditions used in these experiments (—15 per cent protein and ionic strength 0 10) are 25 per cent too low Further correction was made for variation in nitrogen content and refractive index increment among the various plasma protein components Since these corrections increase the  $\gamma$ -globulin concentration of normal plasma from 6 8 to 8 0 grams per liter<sup>43</sup> an additional correctional factor of  $\frac{8.0}{6.8}$  has been applied here The total correction is thus  $1.5 \times \frac{8.0}{6.8} =$



5 The ability of this patient to form certain antibodies was tested in collaboration with Drs M Heidelberger, E A Kabat and A Bendich. The formation of antibodies against pneumococcus polysaccharides was tested in Dr Heidelberger's laboratory by the method he has described.<sup>48</sup> The formation of anti A isohemagglutinin was tested by Drs Bendich and Kabat, using an antigen (A agglutinin) that has been assayed with positive results in 9 normal adults.

### Results

1 A considerable amount of nitrogen, phosphorus and potassium were retained by this patient during the 18 days of the balance study (table 1, fig 5). The concentration of the plasma protein, however, remained the same throughout this period. Table 2 further demonstrates that throughout two years of observation, her plasma protein remained low while her protein intake had been high.

2 Figure 6 shows that this patient initially retained slightly more injected albumin than a normal control of the same age and sex but that the slope of the disappearance curve was parallel to that in the normal subject.

3 The rate of disappearance of the injected  $\gamma$ -globulin as measured by the immunologic methods was rapid, with a half-life of immunologically specific circulating  $\gamma$ -globulin of 3-5 days. The disappearance rate as determined by electrophoresis (table 6) was slower but agreed with the immunochemical results within the limits of error of the electrophoretic method, which is large for the measurement of such a small component.

4 The result of the protein subtraction test was obscured by the fact that the sample taken 24 hours after bleeding was hemolyzed, due to difficulties in venipuncture in this patient; consequently, the extent of the fall of the plasma protein concentration following plasmapheresis could not be evaluated. There was a clear-cut increase of plasma protein 48 hours after bleeding, even more marked than that found in normal individuals (fig 7).

5 The production of antibodies against pneumococcus polysaccharides was definitely weak and no anti-A isohemagglutinin was formed following the injection of an agglutinin.

In summary, one patient (Case 8) failed to elevate her reduced plasma protein level while maintained in positive nitrogen balance by a high protein intake. She showed no defect in her ability to handle injected human albumin and in her ability to regenerate plasma protein following acute withdrawal. Studies on the rate of disappearance of injected  $\gamma$ -globulin were inconclusive. No antibody response was obtained to 2 specific antigens.

### Discussion

Although the presenting features of this syndrome are the marked edema in males and females and ulcers of the legs in males, in no case was the amount of albumin or total protein lowered to the extent that is usually required to produce edema. Therefore, the pathogenesis of this part of the syndrome must be sought elsewhere, such as in a defect of the vascular system, since adrenal and renal mechanisms were found to be intact. In view of the multiple congenital malforma-

tions uncovered in this family, one is tempted to consider a constitutional inferiority of the vascular system. However, systemic humoral mechanisms affecting the vascular system have not been excluded. The lowered oscillometric indices in the arms of 3 females and the markedly hypoplastic veins in one of them tend to strengthen this concept, even though muscle biopsies in 2 patients did not show morphologic vascular changes.

The existence of a clinical entity wherein such cryptogenic edema is coupled with subtle changes in the proportion of electrophoretic components of the blood

TABLE 7—*Blood Groups and Types of the Subjects Studied. Data obtained by Dr. Philip Levine,\* Ortho Research Foundation, Raritan, New Jersey.*

Case No.	Group	Rh†				Negative— Positive +	Remarks‡
		D	C	E	c		
I	O MN	+	+	o	+	+	heterozygous
II H	O MN	o	o	o	+	—	
II L							
II M	O MN	+	+	o	+	+	heterozygous
II N	A MN	+	+	+	+	+	homozygous
3	O MN	+	+	o	o	+	homozygous
4	O MN	+	+	o	o	+	homozygous
6	O MN	+	+	o	+	+	heterozygous
7	O MN	+	+	o	o	+	homozygous
8	O MN	+	+	o	o	+	homozygous
9	O M	+	+	o	+	+	heterozygous
10	O M	+	+	o	+	+	heterozygous
11	O N	+	+	+	+	+	homozygous
12	O N	+	+	o	o	+	homozygous
14	B M	+	+	o	+	+	
15	B MN	+	+	o	+	+	

\* The help of Dr. Levine in our study is thankfully acknowledged.

† Old Nomenclature Rh<sub>0</sub>, Rh', Rh'', H<sub>0</sub>'. New Nomenclature D, C, E, c (On the Nomenclature of the Anti Rh Typing Serums. Report of Advisory Review Board. William B. Castle, Maxwell M. Winterrope and Laurence H. Snyder. *Science* 107: 27-31, 1948.)

‡ Homozygous and heterozygous refer only to the antigenic constitution of the C factor and on statistical probability also to the D factor. No abnormal antibodies were found in the plasma of the wife of this patient 6 months after a stillbirth.

plasma has been established, it is to be expected that more such cases will be found. In this event, the family history should be carefully investigated, as the familial occurrence of this disorder was the most striking feature of this group of patients. The co-existence of congenital malformations, frequent stillbirths (mother Rh positive), and dysproteinemia coupled with constitutional inferiority of the vascular system suggest very strongly the possibility of genetic etiologic mechanisms. This assumption seems even more likely in view of the known hereditary transmission of hemophilia and fibrinogenopenia, both of which appear to be mediated through a lack of certain components of the plasma proteins.<sup>48-49</sup> The hereditary mechanisms governing hemagglutinins, another type of plasma protein component, are well established.<sup>50</sup>

The connection between appearance of edema and puberty, as well as the frequency of stillbirths without Rh immunization (table 7), suggest the possibility of sex hormone disturbance. This may or may not have etiologic or pathogenic significance.

So far, the etiology of the syndrome as well as the pathogenesis of the edema remain obscure. We feel that all known organic causes have been eliminated.

Some information, however, has been gained on the pathogenesis of the disturbed homeostasis of plasma proteins. The pictures observed electrophoretically were (with the possible exception of Cases 7 and 11) striking and significant. In some cases (Cases 6 and 8), the analyses were run in a variety of buffers, so as to exclude changes in mobility of the protein that might be due to an abnormal affinity for citrate iron.

TABLE 8—*Routine Laboratory Findings Obtained in the Hospitalized Patients who Had Dysproteinemia*

Case No	Serum Calcium mg /100 cc.	Serum Inorg Phosphorus	Serum Phosphatases in units		Serum Cholesterol mg /100 cc.			Serum Sodium meq /L.	Serum Potassium meq /L.
			Ac	Alk.	Total	Free	Ester		
5	10.8	3.40	0.46	3.5	—	—	—	143.6	4.5
7	10.9	4.22	—	1.2	159	49	110	139.9	3.9
8	9.8—	3.13—	—	1.8—	115—	49—	66—	137.2—	4.5—
	10.5	4.31	—	4.9	234	107	157	142.0	5.2

	Blood Sugar mg /100 cc.	Hgb Gm /100 cc.	RBC 10 <sup>6</sup>	WBC	Poly %	Ly %	E %	M %
5	95	12.7	—	3850	48	46	3	3
7	90	—	—	—	—	—	—	—
8	63—	12.3—	4.1—	4200—	68—	9—	1—	1—
	105	15.6	4.3	6900	86	26	7	5

In the case of the mother (Case 1), the slight hypoalbuminemia observed at first examination had disappeared on the second examination, nearly a year later. There is some question whether the lowered albumin may not have been due to congestion of the liver, as the patient had signs of cardiac infarction at that time. In Case 6, the marked changes that had been found in two different buffers at the first examination had disappeared on second examination. The patient still had marked ankle edema but the ulcers of the legs present when the first blood had been drawn had healed.

Some of the plasma protein patterns were most unusual. We are aware of only 2 cases in adults that resemble the pattern found in Case 8 (fig. 3). In one case this marked defect of the  $\gamma$ -globulin was on a nutritional basis,<sup>41</sup> and in a second case<sup>42</sup> liver disease was not ruled out and there was a history of chronic alcoholism. Two additional comparable patterns have been reported in children,<sup>43</sup> one of them a definite case of idiopathic hypoproteinemia.<sup>18</sup>

The statistical significance of the changes in  $\gamma$ -globulin in Cases 5 and 8 has been

analyzed in comparison with a group of 13 normal individuals by Dr. J. W. Tukey, Princeton University, and his comments follow.

The 13 normal cases have a mean  $\gamma$ -globulin (in Gm/100 ml) of 0.78 and a standard deviation of 0.106. This compares well with Dole's<sup>54</sup> mean of 0.74 and standard deviation of 0.151 (for 15 cases). If we are prepared to assume that the distribution of amounts of  $\gamma$ -globulin in normal persons follows the so-called normal or Gaussian law, we can set tolerance limits of the form (sample mean)  $\pm \bar{K}$  (sample standard deviation) in such a way that there is a 75 per cent probability that 999 normal cases in 1000 fall between these limits. Values of  $\bar{K}$  are tabulated for different numbers of cases and different probability levels in Eisenhart, Hastay and Wallis<sup>55</sup> at pages 102-107. For 13 cases and the probability levels chosen above  $\bar{K} = 4.059$ .

It seems reasonable to suppose that, while the distribution of  $\gamma$ -globulin may be somewhat skew, with a longer tail toward higher values, the distribution of the logarithm of  $\gamma$ -globulin will be symmetrical, or skewed toward low values. Thus, if we set tolerance limits based on both  $\gamma$ -globulin and on the logarithm of  $\gamma$ -globulin, and then use the outermost limits, we are likely to have a reasonable chance of being conservative. The results are as follows.

*Tolerance Limits for  $\gamma$ -Globulin Concentration in Gm/100 ml based on 13 Normal Cases*

Assumption	Range with 75% probability of covering 999 in 1000
$\gamma$ globulin normally distributed	0.34 to 1.22
Logarithm of $\gamma$ globulin normally distributed	0.43 to 1.37
Conservative recommended	0.34 to 1.37

It will be noticed that the single determination on Case 5 and all 5 determinations on Case 8 fall outside the conservative limits.

The occurrence of such extreme changes is convenient, since by the simple administration of normal plasma one is able greatly to increase the concentration of the deficient plasma component and may then follow its disappearance from the circulation. In Case 8 the injected  $\gamma$ -globulin disappeared rather rapidly and a mechanism seemed to exist that maintained the  $\gamma$ -globulin at its set level far below normal. In an unpublished case of Dr. E. Short, with a sprue-like syndrome, complicated by a history of an earlier disease of the lymphoid tissue, a similar curve of disappearance of  $\gamma$ -globulin was obtained (an autopsy later revealed generalized giant follicular lymphoblastoma). The half-life of the immunologically specific  $\gamma$ -globulin was considerably shorter than the half-life of the glycine labeled  $\gamma$ -globulin as measured by Rittenberg and Shemin (quoted in ref. no. 45). It is impossible for us to offer an interpretation of these facts at the present time.

Evidence was obtained, however, that in one patient (Case 8), a defect existed in the fabrication of circulating antibody against pneumococcus polysaccharide and A agglutinin. A similar defect in synthesis, combined with a homeostatic mechanism set for subnormal levels, may exist for other components. Following the

injection of albumin, its concentration returned to the subnormal preinjection level at a normal rate,\* whereas in Case 7 plasma protein infusions resulted in one instance in a normal protein level for several weeks

In 2 cases the general condition of the patient was improved by the administration of plasma and the edema tended to regress even though the initial hypoproteinemia had been moderate (Cases 7 and 8) In Case 5 no beneficial effect was observed following plasma infusion

The failure of one patient to increase the plasma protein concentration while repleting tissue protein (Case 8) resembled the condition found in hypoproteinemic patients with gastric cancer,<sup>19</sup> tuberculosis<sup>10</sup> and certain types of renal disease<sup>11</sup>

### SUMMARY

1 A new syndrome, *idiopathic familial dysproteinemia*, is described in 4 adult members of one generation, in 2 of their paternal uncles and in 4 members of the second generation The syndrome is characterized by hypoproteinemia and/or abnormalities in the electrophoretic patterns of the blood plasma (dysproteinemia) These are accompanied in the adult by peripheral vascular changes (ulcers of the legs in the men, low oscillometric indices in the women) and edema There are also malformations of the thoracic cage and of the occipital hair distribution in some of the cases

2. The idiopathic nature of the disease was ascertained in some of the patients by study of the nutritional history, of the renal, hepatic and adrenal functions, and of the response to a high-protein diet under controlled conditions

3 In one case detailed studies of the mechanisms of plasma protein regulation resulted in findings that indicate a disturbance in the production of certain protein components The disappearance rate of injected albumin and the rate of replacement of acutely withdrawn plasma protein were normal

4 The clinical and physio-pathologic significance of this syndrome and the possible role of genetic factors are discussed

### APPENDIX CASE HISTORIES

#### (Generation II Fig 1)

1 This white woman aged 70 is the mother and grandmother respectively of some of the other patients herein described

Chief complaint swelling of ankles since age of 50 accentuated in the last five years. There was dyspnea, orthopnea and tachycardia Past History five years ago there was an increase in ankle edema with progressive fatigue dizziness and headaches Her family physician found an elevated blood pressure.

Physical examination B P 135/95 pulse 90 There was a moderately enlarged liver obesity ankle edema and rales in both lung fields Laboratory findings the electrocardiogram showed signs of recent infarction and auricular fibrillation the urines were negative the blood picture was negative The liver function tests were as follows prothrombin time 81 per cent cephalin flocculation and thymol turbidity negative Protein studies have been described above

Course the patient was not hospitalized and was doing well under routine care by her local physician when last heard from

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\* Dr F Albright reports recent metabolic studies in an isolated case of idiopathic hypoproteinemia demonstrating an increased rate of combustion of injected albumin

II H. White woman aged 65, only surviving sister of preceding patient was examined in her home. Her past history was negative. She has 4 children who are in excellent health. Physical examination negative except for a B P of 16/100 which was not causing any symptoms.

II L. An obese white male of 63, one of 3 surviving brothers of husband of Case 1. He was examined at his home and was rather vague about his past history. Twelve years ago he had swelling of the ankles and later an attack of gout. He admitted a fairly high alcoholic intake. Physical examination revealed telangiectasis on face and thorax and pityriasis rosea on thorax. The edge of the liver was palpable 3 finger-breadths below the costal margin in the mid-clavicular line. The floating ribs were markedly prominent.

II M. A white male aged 72, brother of preceding. He had always been in good health except for an episode of pulmonary tuberculosis in early life until 5 years ago when he began to suffer from back pain which became intolerable. Studies at Johns Hopkins and in other university hospitals failed to reveal

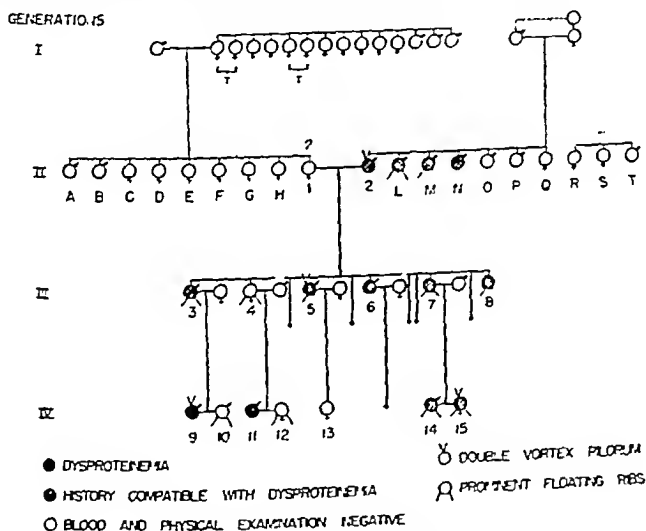


FIG 1—GENEALOGIC TABLE

any enology except osteoarthritis of the spine and an exploratory laminectomy was negative. Physical examination revealed a well-developed and well-nourished white male bedridden with severe back pain. There was a scar of an old lumbar laminectomy and the left wing of the sacrum seemed more prominent than the right and was tender on palpation. There were markedly prominent floating ribs. On the right hand there was a Dupuytren's contracture. The remaining physical examination was negative. Complete laboratory studies could not be done but serum phosphatases and phosphorus were within normal limits.

II N. A third brother of the 2 preceding patients is said to have suffered from edema of the legs during all his adult life. Unfortunately it was not possible to reach him personally at the time of this study.

2. The father of some of the patients described and the husband of Case 1 died at the age of 55 from dropsy following a cholecystectomy. He had edema of the legs at an early age and at times had to use crutches. Repeated vein ligations had to be done for phlebitis of the legs. There are no hospital records to substantiate this history. He had a double vortex pilorum. His family history was not contributory. He had had 6 brothers and one sister and there was a history of tuberculosis at an early age in 2 of these individuals.

*(Generation III Fig. 1)*

3 A white male of 48 tall and thin with grey hair looking somewhat older than his chronologic age. He had had mumps, measles and chicken pox but remembered no diseases in adult life other than an episode of phlebitis 3 years ago with a small ulcer of the leg. Physical examination showed markedly prominent floating ribs, a blood pressure of 120/60 and 1 plus pitting edema of both ankles. The skin over the ankles and the lower part of the calves was thin and atrophic. There was a dark brownish discoloration on the external and internal aspect of the right ankle. Body hair was scant. The remaining physical examination was negative.

4 The history of this well-developed stout woman, sister of preceding patient, was negative. She has enjoyed remarkably good health except for vasomotor disturbances of hands and feet with episodes



FIG. 2.—FAMILY PHOTOGRAPH

of cold and clamminess. Physical examination revealed normal blood pressure but exceedingly low osmometric indices (1-2) in both arms. There was marked prominence of the floating ribs. There was mild edema of the ankles, less evident than in the photograph shown in figure...

5 (MH 8409-Sk 1 163) White man aged 41, brother of the preceding patient. His chief complaint, swelling of the legs, started an unknown number of years ago, and the patient has been using elastic stockings ever since. In 1944 large ulcers appeared on the right lower calf and caused what was termed a deep phlebitis. Saphenous vein ligation was then performed. The right leg has persistently remained more swollen than the left one.

The past history contained a story of swelling of both legs at the age of 8 months, followed by atrophy making walking impossible until the age of 19 months. In the absence of persistent sequelae it was difficult to accept the diagnosis of poliomyelitis then made. There were numerous episodes of infectious

diseases 4 recurrent bronchopneumonias between 1909 and 1911 tonsillectomy in 1915 appendicitis with peritonitis in 1931 The patient gained a great deal of weight from 1939 to 1946 when he weighed 275 lbs He was 6 ft 7 in tall He lost 50 lbs on a reducing diet but regained 20 lbs on the well balanced food intake he had had for several months before admission

Physical examination revealed a white male of tall build He wore shoes size 14 his feet were thus large even for his stature Physical examination was negative except for pitting edema of both legs with brownish discoloration around the ankles bilaterally There was an abdominal scar in the lower quadrant The floating ribs were protruding and there was a double vortex pilorum There was complete edentia The visual acuity was poor bilaterally and there was an early cataract on the right eye (Dr B F Payne) The ophthalmologist found the fundi and visual fields bilaterally normal

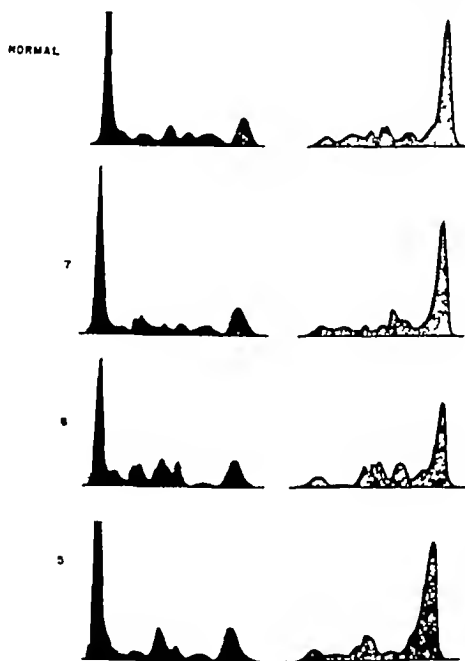


FIG 3—Electrophoretic patterns obtained in veronal-citrate buffer on plasma from a representative normal subject and from three patients 6 corresponds to 6a and 8 to 8b in table 5

Laboratory studies chest x ray was negative and no anomalies were seen in lateral pictures of the skull

Basal metabolic rate several tests were done but the patient was resistant to the procedure and the results while within normal limits were inconclusive

Hematology hemoglobin 12.7 Gm White blood cells 3 850 filamented 44 per cent nonfilamented 1 per cent eosinophiles 3 per cent monocytes 3 per cent lymphocytes 46 per cent

Serology Mazzini tests were negative

Urinalysis urines showed no protein on repeated tests and no other abnormal findings

Gastric analysis showed no free acid before or after histamine Liver function tests these were all negative and the results are shown in table 3

Renal function tests showed no evidence of renal damage (see table 4) Cardiovascular tests blood pressure 125/85 pulse 75 temperature 98.7 Electrocardiograms were negative The oscillometric readings of arms and legs were normal The circulation times by decholin were 20 seconds by ether 15 seconds



The venous pressure was 16 cm of water. Chemical studies of blood are shown in table 7 and are within normal limits. The disturbances of the plasma protein pattern are shown in table 5. In this case there was mild hypoproteinemia and a marked disturbance of the globulin fractions.

Histopathologic studies: serial sections of muscle revealed no morphological anomalies of blood vessels (Dr S. Spitz) (fig. 4).

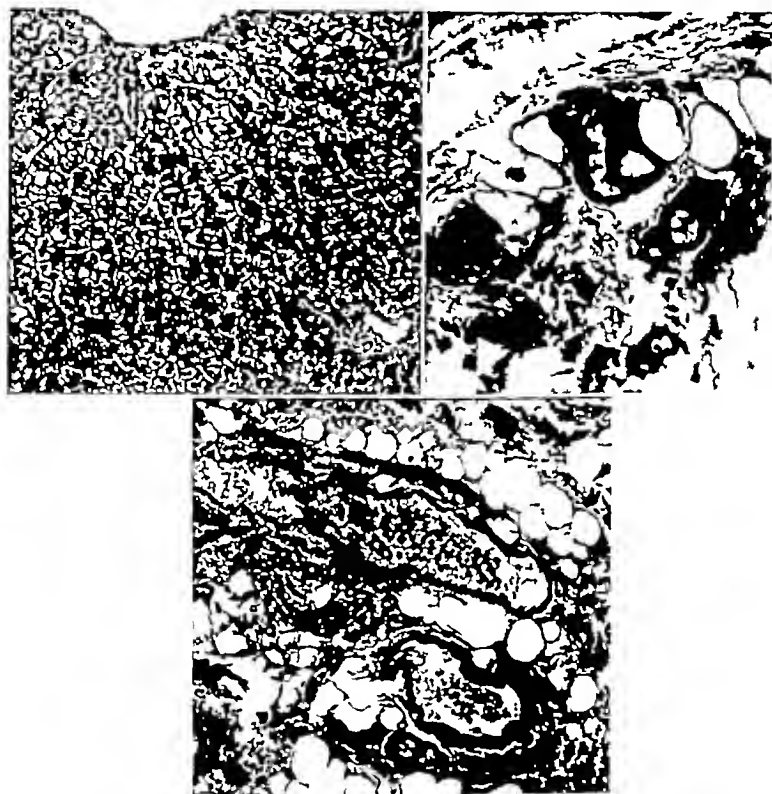


FIG. 4.—*Top*: microphotographs of liver and muscle in Case 8. Note normal appearance of all structures. *Bottom*: biopsy of muscle in Case 5. Normal blood vessels.

**Course of disease.** The patient remained in the hospital for one week only. He left to resume his work as a travelling salesman. The only functional study on his protein metabolism was the administration of 75 Gm. of albumin intravenously. The test could not be carried out completely because of a severe pyrogenic reaction with chills and high temperature. However, about one third of the injected dose was still present in the circulation after 24 hours.

6. This white man aged 38 was seen at his home. He suffered from boils on his head in infancy and had whooping cough at the age of one year. At the age of 8 (1918) he had influenza, followed by chicken pox and roscola. At the age of 15 he had a serious attack of mumps and later on episodes of chronic appendicitis. He was always subject to skin rashes and is sensitive to poison oak. A dark brownish discoloration of both ankles made its appearance at the age of 15. At the age of 20 (1930) the first ulcers of

the leg appeared and kept him bedridden for six months. Ulcers of the legs recurred in 1935, 1938, 1939, 1940, 1943, 1944, 1945, 1946 and 1947, taking each time from some weeks to several months to heal. During a brief period of military service there was an episode of pyuria attributed to infected teeth.

Physical examination revealed brownish discoloration around both ankles, hypertrophy and desquamation of the skin in these areas, prominent floating ribs and no other changes.

Laboratory studies other than electrophoresis of blood were not performed.

7 (MH 86268-SK1 58) This patient is a white married woman of 33. She is the sister of Case 8 and her family history has been described above. She is the mother of 2 children aged 5 and 9 years (Cases 14 and 15).

Chief complaint: swelling of ankles of several years' duration. The patient had been hospitalized for this two years ago and was told that she had a lymphatic condition. She has had measles, whooping cough and chicken pox but like her sister never mumps. She has not lived with her sister for eleven years.

Two years before her admission to this hospital she felt that she was too fat (145 lbs) and reduced on a regime of low dietary intake, dextroline and thyroid. She has continued to take  $\frac{1}{2}$  to 1 gr daily of thyroid since then with no particular indication. Once her weight had stabilized at 132 lbs she returned to a well-balanced and adequate food intake and did not regain her overweight.

There was no history of any disease during her adult life except for the swelling of her ankles which dates back to about the age of 16. This was complicated at one time by phlebitis following an infection of a toe. Systemic review revealed that she had always had cold and clammy extremities, recurrent mild headaches and constipation. Her menstrual history was normal except for menorrhagia for several months following her second pregnancy. Both deliveries were at term and normal.

Physical examination completely negative except for the protrusion of the floating ribs resembling that found in some of the other siblings who were studied and for the marked edema of the ankles. There was also blue discoloration and coolness of the hands and feet. The oscillometric measurements in arms and legs were extremely low.

Laboratory studies: Chest x-rays were within normal limits. X-rays of the bones showed no anomalies. There was a small calcified area in the mid pelvis, possibly a mesenteric node or urethral calcification.

Basal metabolic rate  $-17 \pm 1$   $-20$

Hematology: see table 7

Serology: Mazzini, Kahn and Kline tests negative

Urinanalysis: urines were negative and no albumin was found at any time

Gastric analysis: fasting free acidity was 14 units and total acidity 34 units. This rose following histamine to 55 and 73 units respectively.

Liver function tests: bilirubin, cephalin flocculation, thymol turbidity tests and bromsulphalein retention all gave normal results.

Cardiovascular tests: electrocardiograms were normal.

Circulation times: decholin 16 seconds, ether 13.5 seconds. Venous pressure measured 17 cm of water. The oscillometric indices have been discussed.

Chemical studies of the blood fell within normal limits. The anomalies of the blood plasma proteins of this patient are shown in table 5.

Course of disease: this patient was able to remain in the hospital for only three days. A most remarkable fact was her response to the infusion of 25 Gm of human albumin. Even though no diuresis occurred her ankles decreased markedly in size during the night following the infusion. She was given one liter of human plasma before leaving the hospital and it was rather striking that the total protein level remained above 7 Gm per 100 ml for nearly one month. In spite of this however the ankle edema returned to its previous extent about 2 weeks after discharge.

8 (M.H. 83651-SK1 120) This patient was a white girl of 31, a personnel manager who was born and lived most of her life in the South. She came to work in New York City several years before her admission to Memorial Hospital. Since the age of about 16 she has suffered from swelling of the ankles and legs and from occasional facial edema.

Family history as described above

Past history the patient had the usual childhood diseases except mumps. She had repeated colds, four episodes of pneumonia as a baby and underwent tonsillectomy at the age of 13 which failed to decrease the frequency of colds and sore throats. There is a history of shoulder pains suffered as a small child and the patient still occasionally experiences dull pains in her shoulder girdle.

The systemic review reveals that she suffers from occasional headaches especially whenever she has one of her frequent colds. For a short period in 1944 she had daily elevation of temperature, but no lesions were seen on x ray of the chest and the temperature became normal. She was often rather tired and her swollen ankles were at times attributed to a cardiovascular disorder for which no objective evidence was ever obtained. She has had gingivitis and occasional gastrointestinal upsets. In the past two years, she has had nocturia occasionally once a night and there was usually some urgency for urination. There

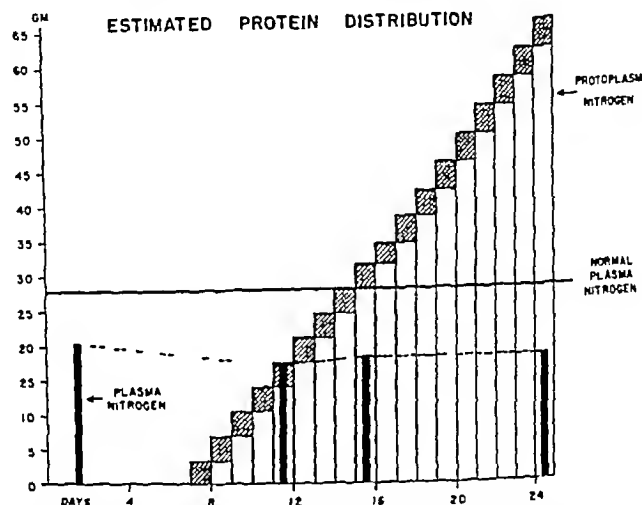


Fig 5—Estimated Protein Distribution Case 8 Cross hatched areas show daily nitrogen retention cumulative nitrogen retention in last column presumably retained for protoplasm synthesis. Solid line at 28 Gm shows normal amount of plasma protein nitrogen (Seven Gm protein per 100 ml. plasma  $\times$  12 Gm nitrogen. This value times normal plasma volume of 2500 ml = 28 Gm.) Full columns show amounts of circulating protein nitrogen found in patient.

was no history or evidence of venereal infection. She started to menstruate at the age of 13 and had a 30-day regular cycle, periods lasting 5 days and slight abdominal pains preceding menstruation with occasional mastodynia at the same time. There was no history of disturbed endocrine function. She was allergic to various foods which caused urticaria to appear; she showed fairly severe urticarial reactions following the administration of plasma. There was no history of severe gastrointestinal or hepatic disorder.

Physical examination revealed a well-nourished white girl of 31 of asthenic habitus. Blood pressure 105/76 pulse 95 temperature 99 F. The skin was moist and warm. In places especially over the upper and lower extremities there was some bluish discoloration (vasodilatation). There was mild seborrhea of the face and scalp. The hair was soft and brown. The finger and toe-nails were exceedingly thin and soft and detached from the nailbed at their tips to a more marked degree than is usually seen; all the nails showed longitudinal ridges.

The bones appeared to be of normal size and configuration except for the floating ribs which protruded more than is usually the case from the thorax. The joints were free from swellings or inflammation and no visible deformities were present.

There was no enlargement of lymph nodes. The ears, nose and throat were normal, the tonsils were absent. No anomalies were found in the eyes or extraocular muscles. The tongue showed slightly atrophic papillae and there was some loosening of the gums from the teeth which were in good repair. The trachea was in the midline. The thorax was symmetrical and clear to percussion and auscultation, the floating ribs protruded unduly. The breasts were small but firm and glandular tissue was distinctly palpable.

The cardiovascular system was normal on physical examination except for the peripheral veins which were extremely small, hardly visible even in infra red photographs or palpable in the antecubital fossae even though there was no excess of subcutaneous fat. There was also striking blue discoloration of hands

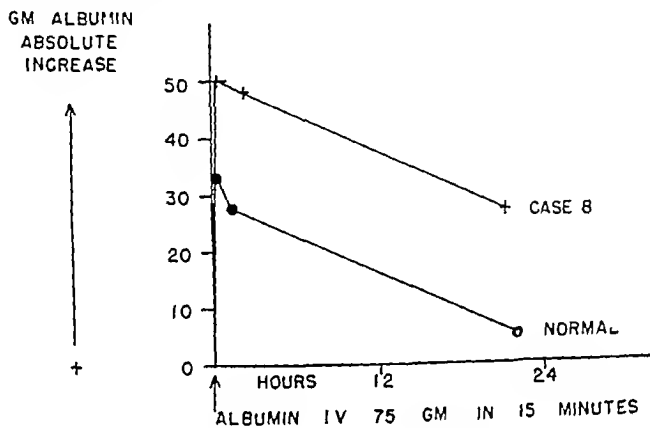
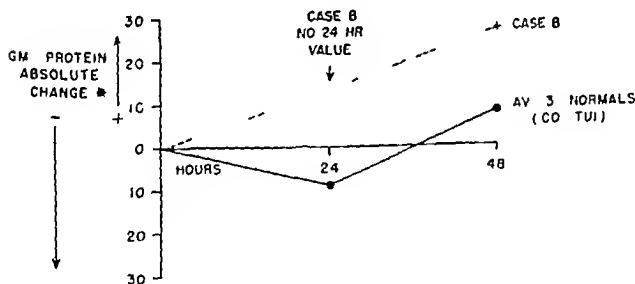


FIG 6—ALBUMIN ADDITION TEST IN CASE 8



\* ASSUMING CONSTANT PV

FIG 7—PROTEIN SUBTRACTION TEST

and feet. The oscillometric examinations of arms and legs showed markedly reduced oscillations, a finding which was to be expected in the edematous legs but which was also marked in both arms. There was pitting edema of both ankles and calves up to the knee.

The abdomen was soft and slightly protruding, there was no tenderness on palpation and no masses were felt. The gynecological examination revealed a normal vulva and vagina, a small uterus in the midline, retroverted and flexed but freely mobile, and normal annexae. The rectal examination was negative. The neurological examination was completely negative.

Laboratory studies: chest x-rays were repeatedly negative. Studies of all bones including the skull showed normal structure and bone age and specifically no signs of decalcification. A gastrointestinal series was negative. The only positive x-ray finding was a congenital lumbarization of the first sacral vertebra.

Basal metabolic rate  $+7$  on two occasions

Hematology from November 1946 to November, 1947 20 blood counts were done. The hemoglobin varied from 12 Gm to 15.6 Gm the red blood cells were 4.1 and 4.3 millions (they were not counted in all blood examinations) The white count varied from 4,750 to 6,900 filamented polynuclear cells from 59 per cent to 81 per cent non filamented forms from 1 per cent to 15 per cent eosinophiles from 1 per cent to 7 per cent monocytes from 1 per cent to 5 per cent and lymphocytes from 9 to 26 per cent. Hematocrits varied between 38 and 41 Metamyelocytes were seen on a few occasions Sedimentation rates were repeatedly normal Blood group and type are shown in table 7

Serology Mazzini Kline and Kahn tests were repeatedly negative Heterophile and Aborts Bang agglutination tests were negative

Urinalysis urines were acid except on one occasion No albumin was ever found either by the routine nitric acid test or with other precipitants such as sulfosalicylic acid trichloroacetic acid heat coagulation etc The centrifuged sediment contained occasional leukocytes and rare epithelial cells never any red cells

Stool examination the appearance of the stools was normal They were well formed, negative for fat blood and undigested muscle fibers No parasites were found The daily fecal nitrogen excretion never exceeded 10 per cent of the intake The fecal fat excretion was below 5 per cent of the intake

Gastric analysis this showed no free acid in a fasting sample and 18 units of total acidity After histamine there were 34 units of free and 40 units of total acidity On another occasion there were 64 units of total and 48 of free acidity following histamine and pepsin was repeatedly found to be present by digestion test

Liver function tests these are shown in table 3 An intravenous glucose tolerance test was normal

Renal function tests shown in table 4 Measurements of renal blood flow glomerular filtration and tubular re absorption were within normal limits Cardiovascular tests electrocardiograms were normal Circulation time by decholin was  $21\frac{1}{2}$  seconds by ether  $10\frac{3}{4}$  seconds The venous pressure was 20 cm. of water The oscillometric studies have been discussed

Tests of adrenal function a Robinson Power and Kepler<sup>6</sup> procedure clearly indicated the absence of Addison's disease The same result was obtained by the Cutler Power Wilder<sup>7</sup> test, as well as by that described by Reforzo-Membrives, Power and Kepler<sup>12</sup> Chemical studies of blood were within normal limits

Histopathologic studies a liver biopsy was performed in local anesthesia through an abdominal incision (Dr G C Child III) and slides showed normal hepatic tissue (Dr S Spitz) A muscle biopsy (rectus abdominis) taken at the same time and examined in serial sections showed no muscular or vascular anomalies

Repeated vaginal smears (Dr A. Carter) showed changes as seen in normal ovulatory menstrual cycle.

The anomalies found in the blood plasma protein patterns are shown above (table 5) The total protein was consistently low (Kjeldahl determinations) and the  $\gamma$ -globulin as measured by electrophoresis was the lowest value seen for that protein in this laboratory

Course of disease since November 1946 this patient has had 5 hospitalizations some for study and one for severe hepatitis probably homologous serum jaundice due to large amounts of plasma given her In the interim between admissions she worked as secretary at the hospital and had her meals from the research diet kitchen Her plasma protein remained low throughout the period of observation exceeding 6 Gm per 100 ml only once following the administration of large amounts of plasma There were two periods during which she had mild temperature elevations in the afternoon for which no cause could be found and which in one instance promptly receded following the administration of 50,000 units of penicillin every four hours for three days The second episode subsided spontaneously during it there was some swelling and reddening over the second joint of the third finger of the right hand accompanied by itching and interpreted by some observers as possibly a rheumatic manifestation by others as a urticarial phenomenon The latter hypothesis seemed more likely as there was no elevation of the sedimentation rate and as the lesion disappeared rapidly under pyribenzamine therapy In the absence of other signs the explanation suggested by some of these febrile and allergic episodes as manifestations of disseminated lupus erythematosus seemed unlikely The patient's course was otherwise uneventful except for the fact shown in table 2, that in spite of high protein intake her plasma protein concentration remained low

On May 2, 1947 the patient left for the South on a low salt high protein diet She returned on June

13 1947, with marked jaundice, anasarca and prostration. Liver functions were disturbed and her plasma protein level was at its lowest point (3.6 Gm per 100 ml). There were ascites and bilateral hydrothorax. Concentrated human plasma and albumin were given and a marked diuresis resulted. There was a dramatic increase of plasma volume and a fall of the extracellular fluid space as measured by thiocyanate. This change was so pronounced that pulmonary edema resulted and had to be treated actively (tourniquets on extremities, morphine). Following the re-establishment of her usual protein level of 5 Gm per 100 ml the patient improved rapidly while on a high protein diet and could be discharged on July 27, 1947. In October, 1947, all liver function tests measured gave normal results.

(Generation IV Fig. 1)

9 (MH 89947-SkI 390) This white boy aged 19 is the son of Case 3. He is a well-developed, healthy individual at present a member of a military academy where he has to undergo a rigid biannual physical examination. He has had chicken pox, mumps, whooping cough and measles. At the age of 7 he underwent a tonsillectomy. He had an injury to his right leg at the age of 12, which healed slowly. Other minor abrasions sustained in the course of sports healed at a normal rate.

Physical examination was negative except for the existence of a double vortex on the occiput.

Liver function tests were negative (thymol turbidity 0.15 ml bromsulfalein 2 per cent bilirubin 0.51 mg [0.29 indirect 0.22 direct 0.11 delayed direct] hippuric acid excretion 1.49 Gm cephalin flocculation negative).

A P S P excretion test was within normal limits.

10 A white boy aged 16, just entering puberty, brother of preceding patient. He had mumps and chicken pox but not measles. In 1939 he suffered an attack of intestinal influenza. Physical examination was negative except for prominent floating ribs.

11 This 13 year old boy had had measles and ascariasis, and has frequent colds. There was mild acne vulgaris. Physical examination was negative.

12 A well-developed white girl of 17, sister of the preceding patient. She had had chicken pox and measles but not mumps. There were no serious illnesses. Physical examination showed marked prominence of the floating ribs. Blood pressure was 125/75 and the oscillometric index in the arms was above 5.

13 This patient could not be reached.

14 This boy, aged 4, is the brother of Case 15 and the son of Case 7. He had a history of repeated colds and refractory infections of the toes. At the time of this examination he was apparently in good health. He had a double vortex pilorum and prominent floating ribs. Blood was taken for electrophoretic analysis. The blood group and type are shown in table 7.

15 This girl of 7 is the daughter of Case 7. She had none of the usual childhood diseases excepting repeated colds. Apparently she has always been in excellent health. Physical examination revealed a well-developed child, with prominent floating ribs and a double vortex pilorum. Blood was taken for electrophoretic analysis. The blood group and type are shown in table 7.

### ACKNOWLEDGEMENTS

We wish to acknowledge the courtesy of Dr. E. Shorr and Dr. E. A. Burkhardt who referred to us the first patient of this group. Several members of the staff of Memorial Cancer Center have helped in this study. The authors wish to thank Drs. E. McCabe and J. B. Trunnell for assistance in the clinical management of the patients; Dr. Aurelia Poror for performing the renal and adrenal function studies; Dr. N. F. Young who supervised laboratory procedures; Dr. H. J. Tagmann who performed the liver function tests assisted by Miss P. Nichols; Dr. C. G. Child III for performing the biopsies; Dr. S. Spitz, for the pathological interpretations and Dr. E. C. Reifstein, Jr. for advice in preparing the graphs and manuscript.

## REFERENCES

- <sup>1</sup> DORLAND W A N The American Illustrated Medical Dictionary, 19th Edition Philadelphia, W B Saunders 1941 P 670
- <sup>2</sup> MYERS W K., AND TAYLOR F H L Hypoproteinemia probably due to deficient formation of plasma proteins study of one case J A M A 181 198-200 1933
- <sup>3</sup> THOMPSON W H McQUARRIE I, AND BELL, E T Edema associated with hypogenesis of serum protein and atrophic changes in the liver with studies of water and mineral exchanges J Pediat 9 604-619 1936
- <sup>4</sup> ALBRIGHT F Cushing's syndrome The Harvey Lectures Series XXXVIII pp 123-186 Baltimore, Williams and Wilkins 1942-43
- <sup>5</sup> BRAOLEY S E AND TYSON C J The Nephrotic Syndrome N England J Med 238 7 223-227 and 238 8 260-266 1948
- <sup>6</sup> COPE O NATHANSON I R ROURKE G M AND WILSON H Management of the Coconut Grove Burns at the Massachusetts General Hospital Metabolic Observations Philadelphia J B Lippincott, 1943 Pp 137-158
- <sup>7</sup> KINSELL L W HERTZ S AND REIFENSTEIN, JR E C Effect of testosterone compounds upon nitrogen balance and creatine excretion in patients with thyrotoxicosis J Clin Investigation 23 880-890, 1944
- <sup>8</sup> CANTAROW A AND TRUMPER M Clinical Biochemistry, ed 3 Revised Philadelphia, W B Saunders 1945 P 339
- <sup>9</sup> HOMBURGER F AND YOUNG N F Studies on hypoproteinemia I Hypoproteinemia in patients with gastric cancer its persistence after operation in the presence of body tissue repletion Blood 3 1460 1948
- <sup>10</sup> Co Tui Personal communication
- <sup>11</sup> KEUTMAN E H AND BASSETT S H Dietary protein in hemorrhagic Bright's disease effect of diet on serum proteins proteinuria and tissue proteins J Clin Investigation 14 871-888 1935
- <sup>12</sup> COPE C L AND GOADBY H K Study of a case of idiopathic hypoproteinemia Lancet 1 1038-1040 1935
- <sup>13</sup> BINORR M W AND KEITH N M General edema of indeterminate etiology Report of three cases J A M A 189 1-6 1937
- <sup>14</sup> JOHANSEN A H Hypoproteinemia Acta Path et Microbiol Scandinav Supp 37 272-289 1938
- <sup>15</sup> RYTAND D A Edema with unexplained hypoproteinemia a syndrome of defective formation of serum proteins in the absence of loss and lack of protein and demonstrable hepatic disease Arch Int Med 69 251-262 1942
- <sup>16</sup> SCHICK B AND GREENBAUM J W Edema with hypoproteinemia due to a congenital defect in protein formation (Case report) J Pediat 27 241-245 1945
- <sup>17</sup> GUTMAN A B AND GUTMAN E B Relation of serum calcium to serum albumin and globulin J Clin Investigation 16 903-919 1937
- <sup>18</sup> PETERMANN M L AND HOGNESS K R Electrophoretic studies on the plasma protein of patients with neoplastic disease I Gastric cancer Cancer, in press
- <sup>19</sup> McCULLAGH E P AND LEWIS L A Tiselius electrophoretic studies of plasma proteins in Addison's disease Am J M Sc 210 81-86 1945
- <sup>20</sup> BING J Studies on proteinuria albuminuria Dissertation Copenhagen 1936 Acta med Scandinav Supp 76 1-151 1936
- <sup>21</sup> MELNICK D AND COWGILL G R Problem of hypoproteinemia Yale J Biol Med 28 49-63 1937
- <sup>22</sup> MATEER J G BALTZ J I MARION D F AND HOLLANDS R A A comparative evaluation of the newer liver function tests—comparison of the intravenous hippuric acid test the cephalin-cholesterol flocculation test the colloidal gold test and a serial bromsulphthalein test with the oral hippuric acid test and the Rosenthal bromsulphthalein test J Dig Dis 9 13-29 1942.
- <sup>23</sup> LEY A B LEWIS J H AND DAVIDSON C S The quantitative determination of the thymol turbidity reaction of serum J Lab & Clin Med 31 910-913 1946
- <sup>24</sup> HANORR F M Flocculation of cephalin-cholesterol emulsions by pathological sera Tr A Am Physicians 53 148-151 1938

- <sup>28</sup> CUTLER, H H, POWER, M H AND WILDER R M Concentration of chloride sodium and potassium in urine and blood, their diagnostic significance in adrenal insufficiency *J A M A* *111* 117-122, 1938
- <sup>29</sup> ROBINSON, F J POWER, M H, AND KEPLER, E J Two new procedures to assist in recognition and exclusion of Addison's disease preliminary report *Proc Staff Meet Mayo Clin* *16* 577-583 1941
- <sup>30</sup> REFORZO-MEMBRIVES J, POWER M H AND KEPLER E J Studies on renal excretion of water and electrolytes in cases of Addison's disease *J Clin Endocrinol* *5* 76-85 1945
- <sup>31</sup> SMITH W W FINKELSTEIN N AND SMITH H W Renal excretion of hexitols (sorbitol mannitol and dulcitol) and their derivatives (sorbitan isomannide and sorbide) and of endogenous creatine like chromogen in dog and man *J Biol Chem* *155* 231-250 1940
- <sup>32</sup> SMITH H W, FINKELSTEIN N ALIMINOSA L CRAWFORD B AND GRABER M The renal clearance of substituted hippuric acid derivatives and other aromatic acids in dog and man *J Clin Investigation* *24* 388-404 1945
- <sup>33</sup> GOLDRING, W, CHASIS H, RANES, H A AND SMITH H W Relations of effective renal blood flow and glomerular filtration to tubular excretory mass in normal man *J Clin Investigation* *19* 739-750 1940
- <sup>34</sup> THORN G W, KOEFF G F LEWIS R A AND OLSEN E F Carbohydrate metabolism in Addison's disease. *J Clin Investigation* *19* 813-832, 1940
- <sup>35</sup> REIFENSTEIN JR E C ALBRIGHT F AND WELLS S Accumulation interpretation and presentation of data pertaining to metabolic balances notably those of Ca P and N<sub>2</sub> *J Clin Endocrinol* *5* 367-395, 1945
- <sup>36</sup> GREGERSEN M I Analysis of colorimetric methods in relation to plasma volume determinations *J Lab & Clin Med* *23* 423-430 1938
- <sup>37</sup> WALLACE, G B, AND BRONIE B B Distribution of administered iodine and thiocyanide in comparison with chloride and their relation to body fluids *J Pharm & Exp Therap* *61* 397-411 1937
- <sup>38</sup> PETERMANN, M L, YOUNG, N F AND HOGNESS K R A comparison of the Howe and the electrophoretic methods for the determination of plasma albumin *J Biol Chem* *169* 379-387 1947
- <sup>39</sup> CONDEN R, GORDON A H, AND MARTIN A J P Qualitative analysis of proteins a partition chromatographic method using paper *Biochem J* *38* 224-232, 1944
- <sup>40</sup> KNAPP E L AND ROUTH J I Electrophoretic patterns of plasma from normal and sick children *Fed Proc Part 2* *6* 267 1947
- <sup>41</sup> HOWE, P E Use of sodium sulfate as globulin precipitant in determination of proteins in blood *J Biol Chem* *49* 93-107 1921
- <sup>42</sup> PILLEMER L AND HUTCHINSON M C Determination of albumin and globulin contents of human serum by methanol precipitation *J Biol Chem* *158* 299-301 1945
- <sup>43</sup> BENDICH A, and KABAT E A Immunochemical estimation of the rate of disappearance of transfused gamma globulin from the blood in two cases of hypoproteinemia *J Lab & Clin Med* *34* 1066-1070, 1949
- <sup>44</sup> PERLMANN G E AND KAUFMAN D The effect of ionic strength and protein concentration in the electrophoretic analysis of human plasma *J Am Chem Soc* *67* 638-641 1945
- <sup>45</sup> ARMSTRONG, JR S H BUDKA M J E AND MORRISON K C Preparation and properties of serum and plasma proteins XI Quantitative interpretation of electrophoretic schlieren diagrams of normal human plasma proteins *J Am Chem Soc* *69* 416-429 1947
- <sup>46</sup> CO TUI BARTTER F C WRIGHT A M. AND HOLT R B Red cell reinfusion and frequency of plasma donations, preliminary report of multiple donations in 8 weeks by each of six donors *J A M A* *124* 331-336 1944
- <sup>47</sup> HOMBURGER F YOUNG N F AND REIFENSTEIN JR E C The protein addition test and the protein subtraction test their relation to hypoproteinemia In preparation
- <sup>48</sup> HEIDELBERGER M MACLEOD C M KAISER S J AND ROBINSON B Antibody formation in volunteers following injection of pneumococci or their type specific polysaccharides *J Exp Med* *81* 303-320 1946
- <sup>49</sup> MACFARLANE R G Boy with no fibrinogen *Lancet* *1* 309-312 1938
- <sup>50</sup> SCHONFOLZER G Die hereditäre Fibrinogenopenie *Dtsch Arch f Klin Med* *174* 406-510 1939



- <sup>48</sup> MacFarlane R G AND SCARBOROUGH H Discussion on factors concerned in blood coagulation and their chemical significance *Proc Roy Soc Med* 38 399-408 1945
- <sup>49</sup> LEWIS J H DAVIDSON C S MINOT G R AND TAYLOR F H L Relation of certain fractions of plasma globulin to the coagulation defect in hemophilia *Blood* 1 166-172, 1946
- <sup>50</sup> LANDSTEINER KARL Specificity of Serological Reactions with a Chapter on Molecular Structure and Intermolecular Forces Cambridge Harvard Press 1945
- <sup>51</sup> KREBS E G Depression of gamma globulin in hypoproteinemia due to malnutrition *J Lab & Clin Med* 31 85-89 1946
- <sup>52</sup> FORBES A P Effect of various types of nitrogen intake and of testosterone propionate therapy on the nitrogen balance and serum protein level of a woman with idiopathic hypoalbuminemia and hypoglobulinemia Conference on Metabolic Aspects of Convalescence Including Bone and Wound Healing February 11-12, 1944 pp 144-147
- <sup>53</sup> STERN K G AND REINER M Electrophoresis in medicine *Yale J Biol Med* 19 67-99, 1946
- <sup>54</sup> DOLE V P The electrophoretic pattern of normal plasma *J Clin Investigation* 23 708-713 1944
- <sup>55</sup> Selected Techniques of Statistical Analysis edited by EISENHART C. HASTAT M W AND WALLIS, W A New York McGraw Hill 1947

# PERIODIC (CYCLIC) NEUTROPENIA, AN ENTITY

## A COLLECTION OF SIXTEEN CASES

By HOBART A. REIMANN, M D and C. THOMAS DEBERARDINIS, M D

ATTENTION was called elsewhere<sup>1</sup> to several peculiar disorders which recur at remarkably regular intervals over many years without otherwise affecting the general health. It is uncertain if these different conditions, including cyclic neutropenia, are unrelated medical curiosities or, more likely, if they have a common cause and can be grouped together as periodic disease. Since the paper was published, a number of other reports of almost identical cases of cyclic neutropenia have been gathered from widely scattered sources, 16 in all. They are listed in approximate order of publication in table 1. A brief résumé of these cases and a detailed report of the study of a patient mentioned in the previous paper are presented here.

A case reported in 1910 by Leale<sup>2</sup> was the subject of two later studies by others. At the age of 3½ months a male infant had attacks of recurrent furunculosis and aphthous lesions in the mouth with fever as high as 40 C (104 F) every few weeks. Leukopenia with 1 per cent polymorphonuclear neutrophile cells was found on several occasions. Malaria was suspected. The case was regarded as recurrent agranulocytosis by Rutledge and his associates<sup>3</sup> in 1930 when the patient was 19. The attacks came at three week intervals with stomatitis, swelling of the cervical lymph nodes, malaise and fever. During the episodes the leukocytes numbered from 2,000 to 4,000 cells with from 0 to 16 per cent neutrophiles and the thrombocytes were diminished in number. The eosinophiles were always increased abnormally especially in an episode. The periodicity of some endocrinologic disturbance was suggested to account for the condition.

Further studies were reported by Thompson<sup>4</sup> in 1934, who tried to establish a relationship of the disorder to the neutropenia known to occur occasionally with the menstrual cycle. The patient by then had developed diabetes insipidus. Measurements of the excretion of gonadotrophic hormone and female sex hormone in the urine showed a fluctuation in rhythm with the neutropenic cycles. It was suggested that the patient, although a man, had a cyclic hormonal disturbance similar to the menstrual cycle. Communication with him in 1945 when he was 34 years old, shortly before death from pneumonia, revealed that the neutropenic cycle persisted at twenty-one day intervals but the constitutional symptoms had disappeared.

Sutton's<sup>5</sup> patient, described in 1911, at the age of 16, had had oral ulcers and fever recurrent every three weeks since the age of 3 months. A single normal leukocyte count was recorded during a free period. The case was again reported by Hoxie<sup>6</sup> two years later when the episodes recurred every fourteen to twenty days. At that time, pain and swelling of his left knee and occasionally of the hands oc-

curred during some of the episodes, at four to eight week intervals. At one occasion, 35 cc of clear fluid was aspirated from the knee. All of his teeth were removed as possible foci of infection with no effect on the episodes. No other leukocyte counts were recorded.

TABLE I — *Published Cases of Cyclic Neutropenia*

Author	Sex	Age at Onset	Age at last Examination	Interval in days	Fever	Other Remarks
1. Leale Rutledge Thompson	M	3½ mo	34	21	+	Psychic disturbance, eosinophilia at times
2. Sutton Hoxie	M	3 mo	18	14-21	+	Swelling of left knee at times every 4-8 weeks
3. Doan	F	1	35	21	0	Leukopenia less after splenectomy
4. Embleton	F	16	43	17-20	+	Series of episodes in 1919 1926 1936
5. Plum	M	2	12	21	+	
6. Imerslund	M	1	16	19-23	+	
7. Vahlquist	F	2 mo	4	21-22		Father had leukopenia of 3,000 cells but no other symptoms
8. Reznikoff	M	1	18	21	+	Abdominal pain. Concomitant cyclic diminution of 17 ketosteroids. After splenectomy leukopenia not so striking symptoms persist
9. Barling	F	12	32	21-28		Eosinophilia 1-10%. Episodes continued during pregnancy
10. Löffler	M	24	30	21	+	Eosinophilia arthralgia. Called Felty's Syndrome. No benefit after splenectomy
11. Reimann	M	5	21	20-25	0	Arthralgia
12. Alt	M	2	14	18-22		
13. Rulland	F	6 mo	8	21	+	Excretes abnormally large amounts of gonadotrophins
14. Fullerton	M	62	63	23-28	+	Splenectomy relieved symptoms but cyclic neutropenia occurs to lesser degree
15. Erf	F	52	56	21	+	Arthralgia splenectomy called Felty's Syndrome. Oral ulcers ceased after splenectomy. Still has cyclic low grade leukopenia
16. Owren	M	18	23	14-21	+	No benefit after splenectomy

Doan's<sup>7</sup> patient, a woman of 18, had had neutropenia, dermal and oral ulcers every eighteen to twenty-one days since the age of 1. The patient and her mother had hemolytic icterus. During the episodes there was absolute neutropenia and a total leukocyte count of 2,000 to 3,000, of which 50 per cent were monocytes. After splenectomy the leukocytes did not fall below 5,000 per cubic millimeter but the number of granulocytes continued to fluctuate in the usual rhythm but to a lesser extent.

Embleton's<sup>8</sup> patient, a woman of 43, had ulcers in her mouth, malaise and fever of 38.9 C (101 F) every seventeen to twenty days for several months in 1919, in 1926 and when reported in 1936. In the intervals between the episodes she felt well and studies of her blood showed no abnormality. During the episodes, the leukocyte count fell to 3,000 per cu mm and the neutrophil cells disappeared. The erythrocytes showed vacuolization, poikilocytosis and many were microcytes, and the platelets 'became exceedingly numerous'. To account for the cycles, the author raised the question of a response to the life cycle of some parasite.

Plum's<sup>9</sup> patient, a youth of 12, had had recurrences of neutropenia at three week intervals since the age of 2.

A patient, aged 16, studied by Imlerslund<sup>10</sup> had had recurrences of shivering, malaise, anorexia, fever of 41 C (105 F), and swollen cervical lymph nodes every three weeks since the age of 14 months. During observation, the number of leukocytes did not fall greatly during the episodes, but the neutrophil cells diminished to 1 to 6 per cent. The sternal marrow during an episode showed a shift to the left, hyperplastic reticulum, an arrest of the development of neutrophil cells at the myelocyte-promyelocyte stage, and an increased number of monocytes compatible with the picture seen in malignant neutropenia. Injection of epinephrine hydrochloride during the neutropenic period caused the lymphocytes to increase, not the neutrophils. While an endocrinologic basis was suspected as an underlying cause of the cyclicity of the attacks, there was no clinical evidence of endocrine dysfunction and a normal amount of folliculin was excreted.

In Vahlquist's<sup>11</sup> patient, a girl of 4, episodes of cutaneous abscesses, fever and cervical lymphadenopathy began at the age of 2½ months. Oral ulcers are not mentioned. The recurrences appeared at fifteen to forty-five day cycles, but the basic rhythm was twenty-one to twenty-three days. Mild asthma occurred at times. Monocytosis compensated for extreme neutropenia. The total leukocyte count often fell to 2,900 in the episodes. The bone marrow obtained during an attack suggested myeloblastic leukemia rather than agranulocytosis. The leukocytes of the father numbered 3100 and 4600 on two occasions, but no leukopenic rhythmicity was demonstrated.

Reznikoff's<sup>12</sup> patient, a youth of 18, had had periodic attacks of fever, canker sores and abdominal pain since infancy. The episodes occurred every twenty-one days and lasted ten days, of which four were usually spent in bed. The cervical and axillary lymph nodes and the spleen became swollen during the attacks. The leukocytes, normal during the free intervals, dropped to 3,000 and 2,000 cells per cu mm and the neutrophil cells to 2 to 15 per cent of the total. Between attacks, the leukocyte count of the marrow varied between 19,000 and 56,000, of which 13,500 to 37,500 were neutrophil cells or their precursors. At the low point the count fell to 1,800 with only 340 neutrophilic elements. Over a three month period the 17-ketosteroid excretion showed a diminution after the onset of the episodes. Other studies including those to determine an allergic disturbance were unrevealing. After splenectomy the only changes noted were that the total leukocyte count did not fall so far as before and the abdominal pains were not so severe.

The case briefly reported by Barling<sup>13</sup> occurred in a woman of 37, who since the

age of 12 had had ulcers in the mouth at intervals of three to four weeks. The ulcers were worse during pregnancy in 1944. Examination of the blood during the free periods showed no abnormalities except for a persistent slightly low percentage of neutrophile cells. During the episodes the total count fell to 1300 at times and the percentage of neutrophilic cells to 15.

The patient studied by Löffler and Maier<sup>14</sup> was regarded as having Felty's syndrome with cyclic agranulocytosis because of anemia, arthralgia, lymph node swelling and granulocytopenia. He was 30 years old. After an attack of pneumonia six years before in 1942, leukopenia and monocytosis had been detected. The spleen and lymph nodes enlarged slightly and stomatitis was noted. He was well, however, until seven months later when polyarthritis lasting three days occurred. The leukocytes numbered 4,600 with 15 per cent neutrophile cells. In the next fourteen months there occurred periodic attacks of polyarthritis with fever at regular twenty-one day intervals. The neutrophiles began to diminish to almost complete agranulocytosis five days before fever appeared. At the time, the cells of the marrow showed a predominant promyelocytic picture. Splenectomy brought no benefit. Examination of the spleen showed chronic inflammatory hyperplasia with eosinophilia.

During the course of observation, aortic insufficiency, presumably from endocarditis, developed in 1943 and shortly after, an attack of pneumonia and empyema. His tonsils and all of his teeth were removed as presumed foci of infection, with no beneficial effect.

Dr. Howard Alt, of Chicago, kindly supplied the following data of his patient. A youth of 18 had had attacks of gingivitis and oral ulcers since the age of 2. He was studied over a five months period in 1941-42 during which six episodes with almost complete neutropenia were observed. They came at intervals of eighteen to twenty-two days. The monocytes increased to 30 to 36 per cent during the periods and the total leukocyte count was 4,000. In the free periods the neutrophiles comprised from 30 to 50 per cent of the leukocytes.

Dr. C. F. Rolland, of Edinburgh, informed us of his patient, a girl aged 7½, who has had febrile attacks with oral ulcers at intervals of about twenty-one days since 6 months of age. There was neutropenia at all times which became absolute during the episodes when monocytosis occurred. An endocrinologic basis of the disorder was suspected and measurements showed the excretion of larger amounts of gonadotrophic hormones than normal.

A woman of 56, studied by Erf,<sup>15</sup> had weakness, fatigue, fever and oral ulcers at three week intervals for over two years. Her menopause occurred at the age of 50. She was studied at this hospital preparatory to splenectomy. There were ulcers on the mucosa of the tongue and cheeks, and the spleen was palpable. Fever was present for two days. The leukocytes numbered between 1,000 and 2,000 with 2 to 4 per cent polymorphonuclear cells. After splenectomy the symptoms disappeared but the leukocytes (counted elsewhere) at times numbered less than 5,000 with 1 to 25 per cent neutrophile cells.

According to Fullerton and Duguid,<sup>16</sup> the disorder began in their male patient at the age of 62 and recurred at intervals of twenty-three to twenty-eight days.

The episodes were characterized by sore throat, conjunctivitis, ischio-rectal infection and oral ulcers. Between attacks the neutrophils rarely reached the normal number. The fall in the number of neutrophils in an episode preceded the rise of temperature and they usually disappeared for four or five days. Studies of the marrow, as in our patient, indicated a periodic failure of the production of neutrophil cells as the cause of their disappearance. No fluctuation in the excretion of 17 ketosteroids was demonstrable. Sulfonamide compounds, penicillin, antihistaminic drugs and pyridoxine had no effect on the condition. After splenectomy, distressing symptoms no longer occurred, but as in Doan's and Reznikoff's patients, cycles of neutropenia recurred in lesser degree of severity.

Owren's patient,<sup>17</sup> a man of 23, had had episodes every two to three weeks since the age of 18, often with complete agranulocytosis. They lasted a week,

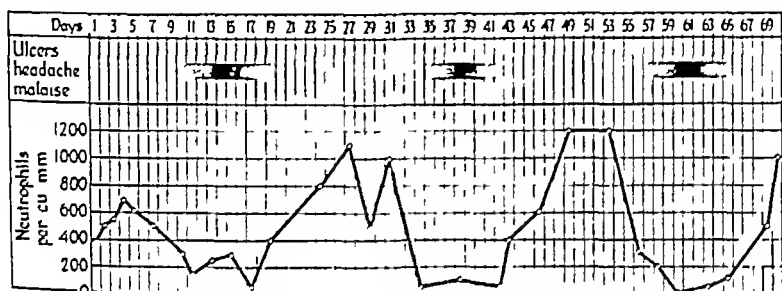


FIG. 1.—Cyclic neutropenia, oral ulcers, headache and malaise lasting eight to ten days and recurring every twenty-three to twenty-six days. The diminution of the neutrophil cells precedes the clinical symptoms represented by the shaded bars to indicate their gradual increase and decrease in intensity.

were accompanied by fever, oral ulceration, and at times by conjunctivitis, ulceration of the skin of the face, perineum and extremities. Studies of the marrow showed disappearance of the mature granulocytes during an episode and their rapid reappearance before the neutrophils returned to the peripheral blood. Between the attacks, the granulocytes in the blood seldom exceeded 1000 per cubic millimeter. Tonsillectomy and splenectomy had no effect on the condition and the patient died later from pneumonia.

#### CASE REPORT

**L. F.** a man aged 23, had been studied at a hospital when 5 years of age for painful swollen joints, general aching, enlarged cervical lymph nodes and sores in his mouth. A diagnosis of rheumatic fever was made. The disorder recurred a year later and after that two or three times a year. He was well otherwise.

In 1944, at the age of 18, he entered the Army. In 1945 he was treated at a military hospital for acute tonsillitis, but no laboratory studies were made. In June 1945, the characteristic three-week cyclic attacks of fever, headache, neutropenia, malaise, sore throat, swelling of the cervical lymph nodes, arthralgia and oral ulcers began. Therapy with sulfadiazine and penicillin had no effect on the condition.

He was discharged from the Army and entered the Jefferson Hospital for study as a patient of Dr. L. M. Tocantins in December 1945. He was observed for nine months. A portion of a chart showing the fluctuation of the number of neutrophil cells in relation to the oral ulcers is shown in figure 1. The

episodes recurred in twenty to twenty-six day cycles. An episode began with a gradual diminution in the number of neutrophile cells for three to four days when they occasionally disappeared from the blood. During this period the signs and symptoms began at times accompanied with fever of 37.4 C (100 F). He usually remained ambulatory but in some attacks had to go to bed. Physical examination revealed a healthy looking well-developed man with a small ulcer in his tongue, a reddened pharynx and slightly swollen anterior cervical lymph nodes. The edge of the tongue was scalloped with scars from previous ulcers. The temperature was normal and the liver and spleen were not palpable.

The erythrocytes numbered 4,100,000, the hemoglobin 14 Gm. There were 3,700 leukocytes of which 15 per cent were neutrophiles, 39 per cent lymphocytes, 43 per cent monocytes and 3 per cent eosinophile cells. The sedimentation rate was 21 mm in 60 minutes, the hematocrit 41 per cent, the bleeding time 30 seconds. The erythrocytes were normally fragile to hypotonic salt solutions. The venous clotting time and clot retraction were normal. The serologic tests for syphilis gave negative results. The Van den Bergh test gave a negative direct reaction and the indirect reading was 0.8 mg. A leukocyte count made between 2 episodes showed 5,000 cells of which 22 per cent were neutrophiles. There was a constant compensatory increase in the number of monocytes. The other cellular elements remained fairly unchanged in number.

Repeated studies of the sternal marrow showed granulocytic hypoplasia during the episodes. Between times these elements were normal. Changes in the marrow always preceded those in the blood. All of the other usual laboratory studies and roentgenograms gave negative results. Many measurements of the basal metabolic rate gave normal results during and between episodes. No cyclic changes were noted in measurements of the CO<sub>2</sub> combining power of the blood over a period of thirty days. The excretion of urinary gonadotrophins, estrogens and 17 ketosteroids were measured thirteen times without evidence of synchronous fluctuation with the cycles of neutropenia. On all occasions the amounts were within normal limits.

A number of special tests were performed during the episodes and in the free periods. In an interim period 25 mg. of adrenocorticotrophic hormone was injected intramuscularly and the leukocytes counted at hourly intervals. A normal response occurred. The number of leukocytes remained constant but the percentage of neutrophile cells rose from 59 to 80 by the fourth hour, the highest ever noted in this person with a corresponding diminution of monocytes. The total number of eosinophiles fell from 166 to 53 per cu. mm. The test was repeated during an episode of neutropenia. The eosinophile cells again were diminished by 50 per cent but the leukocyte count fell from 4,000 to 2,000 with a further decrease in the neutrophile cells and an increase in lymphocytes and monocytes.

During another interim period the intravenous injection of 0.1 unit per kilogram of crystallin insulin caused a slight increase in the number of leukocytes but with no increase in the number of neutrophile cells.

During two periods of neutropenia 1 cc. of 1:1000 solution of epinephrine hydrochloride was injected subcutaneously. Leukocyte counts made at five minute intervals thereafter revealed no changes in the number of component cells on either occasion. Either too few cells were available for release into the blood or they failed to be released.

Brewer's yeast in amounts of 25 tablets daily together with the intramuscular injection of 15 units of liver extract three times a week had no effect on the cycles. Folic acid in doses of 100 mg. orally daily or 20 mg. given intramuscularly, yellow bone marrow in amounts of 1 cc. intramuscularly every other day and pyridoxine in doses of 200 mg. intravenously daily given successively over adequate periods all had no effect on the episodes. Testosterone propionate 50 mg. intramuscularly twice a week given over a period of three weeks was likewise ineffectual.

## DISCUSSION

Two striking features stand out in the 16 cases observed. One is the similarity of all cases sufficient to warrant establishment of the disorder as an entity, the other is the remarkably uniform three week regularity of the cycles. In 9 instances the disorder began in infancy and in 2 at ages 5 and 12. Two began, or became evident, at ages 56 and 62. In most instances the recurrences when once established,

persisted, but in one,<sup>8</sup> the episodes recurred in three distinct periods. Oral ulcers suspected as the cause by some, probably represent only the secondary effects of other disturbances as in the better known forms of neutropenia or agranulocytosis. Fever noted in most patients may be caused by the mild infection incident to the ulcers, as suggested also by cervical lymphadenopathy. It is noteworthy, however, that in only 2 patients,<sup>14, 16</sup> were severe infections recorded, in contrast with their frequency in other forms of severe neutropenia. Either the cyclic neutropenic stage does not last long enough to allow serious infection to occur or neutrophil cells are not the most important factor in the defense against infection. Arthralgia is recorded in 4 cases, prominent enough in 2<sup>14, 16</sup> to suggest a diagnosis of Felty's syndrome. Two patients<sup>4, 17</sup> died from pneumonia.

Ten of the 16 patients were males. The frequency with which the disorder begins in infancy suggests a congenital aspect. In one instance<sup>11</sup> a genetic influence is suggested by the discovery of leukopenia without other signs or symptoms in the patient's father.

It is highly probable that the disorder is not so rare as it seems to be from the few cases thus far reported. They may represent only the severest instances of an unrecognized cyclic condition which could be discovered only if the leukocytes were counted frequently in many persons over long periods, particularly in the relatives of patients with the disorder. That other cases, unrecognized as such, exist is suggested in queries addressed to the editor of a medical journal.<sup>18</sup> In one instance, a man aged 21 had recurrent oral ulcers lasting seven to ten days at intervals of two weeks to three months for three years. In another, a woman, aged 27, had recurrent episodes of sore throat at intervals of two to four times a month for many years. A relation of cyclic neutropenia to other periodic disorders such as periodic fever, benign paroxysmal peritonitis and intermittent arthralgia as suggested elsewhere<sup>1</sup> is likely. The length of the cycles is about the same in each, and certain clinical features such as leukopenia, fever, arthralgia and abdominal pain are present at times in all four conditions. Demmer's<sup>19</sup> case of a man aged 61, who had recurrences of purpura and thrombopenia at twenty-eight day intervals for six years, may also fall into the group.

The cause of the disorder and why the cycles are of three weeks' duration in each case are obscure. To some observers, the rhythmicity suggests a hormonal influence, yet there is no relationship of the episodes to the menstrual cycle, they occur in both sexes, they may commence in infancy or after the menopause, and except for diabetes insipidus in Thompson's patient, no other endocrinologic disturbance is evident in any of the 16 cases. Synchronous cyclic fluctuation of measurable hormones, even if demonstrable, may be the result of the disorder, not a cause. Hormonal therapy failed to influence the cycles in our patient and in those of others. The attacks persisted during pregnancy in one instance.<sup>12</sup>

The cycles are not likely to be connected with a normal periodic renewal or formation of neutrophil cells since the change in all cases is that of diminution in number. The neutropenia probably results from a decrease in the rate of formation as suggested by changes in the bone marrow preceding those in the blood. Eosinophilia, noted in 3<sup>4, 13, 14</sup> may represent a compensatory increase of the



cells or some allergic reaction. Asthma, however, is recorded in only one instance<sup>11</sup> and antihistaminic drugs had no effect on the cycles.<sup>16</sup> It is most unlikely that any infection would cause changes to occur so regularly and uniformly over periods of years.

Of the various forms of therapy thus far applied, only one caused a significant change. In 4 cases,<sup>7, 1\*, 16, 18</sup> splenectomy induced an amelioration of the symptoms or a less striking diminution of the neutrophils, or both, but in two cases<sup>14, 17</sup> no benefit followed. In Thompson's case,<sup>4</sup> symptoms disappeared spontaneously but the neutropenic cycles persisted.

### SUMMARY

Sixteen cases of an entity, periodic neutropenia, have been collected. They are characterized by remarkable clinical uniformity and regular recurrences of neutropenia at three week intervals. The entity may be a variant of a larger group of periodic conditions. The cause is unknown.

### REFERENCES

- <sup>1</sup> REIMANN H. A. Periodic disease. A probable syndrome including periodic fever, benign paroxysmal peritonitis, cyclic neutropenia and intermittent arthralgia. *J. A. M. A.* 136: 239-244, 1948. Periodic disease. Periodic fever, periodic abdominalgia, cyclic neutropenia, intermittent arthralgia, angio-neurotic edema, anaphylactoid purpura and periodic paralysis. To be published *ibid.* 140 (Suppl.) 1949.
- <sup>2</sup> LEALE, M. Recurrent furunculosis in an infant showing an unusual blood picture. *J. A. M. A.* 54: 1845-1855, 1910.
- <sup>3</sup> RUTLEDGE B. H., HANSEN PRÜSS O. C. AND THAYER W. S. Recurrent agranulocytosis. *Bull. Johns Hopkins Hosp.* 46: 369-389, 1930.
- <sup>4</sup> THOMPSON W. P. Observations on the possible relationship between agranulocytosis and menstruation with further studies on a case of cyclic neutropenia. *New England J. Med.* 210: 176-178, 1934.
- <sup>5</sup> SUTTON R. L. Peradenitis mucosa necrotica recurrens. *J. Cut. Dis.* 29: 65-71, 1911.
- <sup>6</sup> HOXIE G. H. Pyorrhea due to organisms other than amebas. *J. A. M. A.* 65: 1908-1909, 1915.
- <sup>7</sup> DOAN C. A. The neutropenic stage. *J. A. M. A.* 59: 194-204, 1932.
- <sup>8</sup> EMBLETON D. Rhythmic agranulocytosis. *Brit. M. J.* 2: 1258-1259, 1936. *Proc. Roy. Soc. Med.* 31: 980-982, 1936-37.
- <sup>9</sup> PLUM P. Clinical and Experimental Investigation in Agranulocytosis. Dissertation, Copenhagen 1940. P. 410.
- <sup>10</sup> IMERSLUND O. A case of agranulocytosis. *Acta Paediatr.* 30: 232-250, 1942. Suppl. 1.
- <sup>11</sup> VAHLQUIST B. Cyclic agranulocytosis. Report of a case with a short survey of the disease. *Acta Med. Scand. Suppl.* 170: 531-542, 1946.
- <sup>12</sup> REZNIKOFF P. Cyclical neutropenia. A case study with bone marrow findings before and after splenectomy. *Tr. A. Am. Physicians* 59: 276-281, 1946.
- <sup>13</sup> BARLINO B. Chronic cyclic granulopenia. *Proc. Roy. Soc. Med.* 61: 653-654, 1948.
- <sup>14</sup> LÖFFLER W. AND MAIER C. Über einen Fall von Feltyschem Syndrom mit cyclischer Agranulocytose. *Cardiologia* 12: 195-210, 1947-48.
- <sup>15</sup> ERF L. A. AND FRY K. E. Primary splenic neutropenia. *Am. J. Clin. Path.* 19: 48-50, 1949.
- <sup>16</sup> FULLERTON H. W. AND DUQUOID H. L. D. A case of cyclical agranulocytosis with marked improvement following splenectomy. *Blood* 4: 269-277, 1949.
- <sup>17</sup> OWSEN P. A. Cyclic agranulocytosis. *Acta Med. Scand.* 134: 87-97, 1949.
- <sup>18</sup> Recurrent ulcers of the mouth. *Queries and Minor Notes J. A. M. A.* 136: 433, 1948. Recurrent pharyngitis. *ibid.* 133: 358, 1947.
- <sup>19</sup> DEMMER T. Morbus Maculosus Werlhofii in regelmässigen vierwöchentlichen Schüben bei einem 60-jährigen Mann nebst Untersuchungen über Blutplättchen. *Folia haemat.* 26: 74-86, 1910-1911.

## LIVER EXTRACT REFRACTORY MEGALOBlastic ANEMIA

By JOHN F. MUELLER, M.D., V. R. HAWKINS, R.N., AND RICHARD W. VILTER, M.D.

RECENTLY we have had the opportunity to study a patient with a macrocytic anemia and megaloblastic bone marrow, who was refractory to parenteral therapy with vitamin B<sub>12</sub> and refined liver extract, but who responded to folic acid. This type of macrocytic anemia is rare, particularly in this country. Observations made in this case support the concept of a chemical interrelationship between liver extract, vitamin B<sub>12</sub> and folic acid and contribute evidence favoring the existence of another factor necessary for normal erythrocyte maturation.

### CASE REPORT

L. R., a 52-year-old former railroad yard worker was referred to our out-patient department on 9-17-48 by a private physician for the treatment of pernicious anemia with vitamin B<sub>12</sub>. The patient gave a history of about six months' duration of progressive weakness, shortness of breath on exertion, several attacks of syncope and sore tongue. The latter prevented the patient from eating most solid foods. Increasing drowsiness, failing vision and increased sensitivity to cold were other subjective complaints. There was no history of diarrhea at any time. His diet prior to this illness had probably been adequate but he admitted a rather heavy intake of alcohol over a period of years. His family had noted a definite change in his personality manifested by extreme irritability and ill humor.

Physical examination revealed a thin, pale white man who appeared chronically ill. He cooperated well. His sclerae were not icteric but his tongue was atrophic and reddened along the lateral margins. His chest, heart and abdomen were normal. All the deep tendon reflexes were extremely hyperactive but there was no clonus nor pathologic reflexes. The Romberg test was normal. Perception of vibration was reduced in the lower extremities, more over the right ankle than the left. Position sense was intact in the toes. There were no other sensory abnormalities.

Laboratory examination revealed: erythrocyte count 1,620,000 per cu. mm., hemoglobin 6.8 Gm. per cent, hematocrit 20 per cent, reticulocytes 1.9 per cent, M.C.V. 123 cu. microns, M.C.H. 4.2 micrograms, M.C.H.C. 34 per cent, white blood cell count 3550 per cu. mm. with a normal differential count, and platelets were 181,440 per cu. mm. A gastric analysis done by the private physician revealed a histamine fast achlorhydria. Urinalysis Kahn, E.C.G., barium studies of the upper and lower gastrointestinal tract, x-ray of the chest and brucella agglutination were normal. A needle biopsy of the sternal bone marrow revealed maturation arrest in the erythrocyte series at the megaloblastic and early erythroblastic stages of development. Bizarre metamyelocytes and macrocytic polychromatophilic normoblasts were abundant. (See table 1.)

We concurred in the diagnosis of pernicious anemia and administered 8 micrograms of vitamin B<sub>12</sub> parenterally (we have obtained maximal reticulocyte responses with only 4 micrograms of vitamin B<sub>12</sub>). Thereafter reticulocytes were counted each day. Erythrocytes and hemoglobin were determined every third day. Reticulocytosis did not occur. By the fourth day after therapy the patient was complaining bitterly about the soreness of his tongue and small congested papillae were visible on the smooth lateral margins. He was placed on multivitamin tablets without relief. Neurologic examination did not change. By 9-27-48, ten days after the initial dose of vitamin B<sub>12</sub>, the erythrocyte count had dropped to 1,185,000 per cu. mm., the hemoglobin to 6 Gm. per cent and the hematocrit to 17 per cent. He was then given 8 micrograms of vitamin B<sub>12</sub> on each of three successive days. Although there seemed to be a little tempo-

From the Department of Internal Medicine, University of Cincinnati, Cincinnati, Ohio.  
These investigations were aided by grants from Merck and Co. Inc., Rahway, New Jersey and the Robert Gould Research Foundation, Inc. We wish to thank Merck and Co. Inc. for their liberal supply of vitamin B<sub>12</sub> and Lederle Laboratories, Inc. for the folic acid.

rary subjective improvement the patient continued on a downhill course and was admitted to the Cincinnati General Hospital on October 2, 1948 for further study and treatment

# L.R., 52 w<sup>o</sup>, REFRACTORY MEGALOBlastic ANEMIA

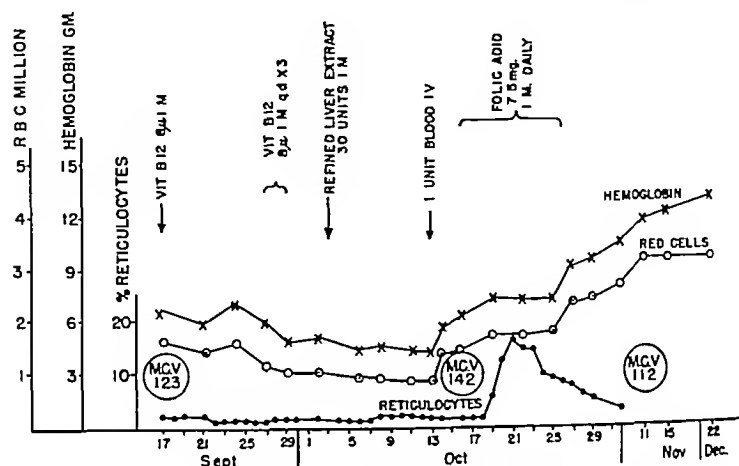


FIG. 1—THE HEMATOLOGIC COURSE OF A PATIENT WITH REFRACTORY MEGALOBlastic ANEMIA

TABLE 1—Bone Marrow Counts on a Patient with Liver Extract Refractory Megaloblastic Anemia

Date	9-17-48 On Admission	10-3-48 After Vitamin B <sub>12</sub>	10-13-48 After Liver Extract	10-26-48 After Folic Acid
Polymorphonuclear neutrophile	48	37	38.5	57
Metamyelocyte	14.5	33.5	29.5	24
Myelocyte C	8.5	3	8.5	2
Myelocyte B	3	3.5	5	0.5
Myelocyte A	1	3	1	0
Myeloblast	0	0.5	0	0
Lymphocyte	12.5	10	12	10
Young lymphocyte	0	0	0	0
Monocyte	0.5	0	0.5	1
Young monocyte	0.5	0	0	0.5
Eosinophile	2	2	2	0.5
Eosinophilic myelocyte	1.5	2	1	2.5
Basophile	0	0	0.5	0
Basophilic myelocyte	0.5	0	0	0
Plasma cell	4.5	1.5	1.5	2
Clasmatocyte	0	0	0	0
Primitive cell	3	4	0	0
Megaloblast	4	7	1.5	0
Early erythroblast	10	13	18.5	1.5
Late erythroblast	19.5	10.5	33	14
Normoblast	12.5	45	24	46
Myeloid Erythroid ratio	2.1	4.3	5.4	5.3

On his admission the physical examination gave essentially the same findings except for a small papule on the margin of his tongue which later broke down to form a small ulceration. The neurologic examination was unchanged. The erythrocyte count was 1 050 000 per cu mm, hemoglobin 5 Gm per cent, hematocrit 16 per cent, white blood cells 1950 per cu mm, reticulocytes 1 per cent. Liver function tests were normal except for retention of 8.5 per cent of injected bromsulphthalein (5 mg/Kilo) after forty five minutes. The total serum bilirubin was 0.4 mg per cent. An analysis done on a 24 hour stool specimen revealed a total fat content of 16 per cent by dry weight, 7.9 Gm total fat in twenty four hours of which 5 Gm were fatty acids. Oral glucose tolerance test revealed slow submaximal absorption but not a flat curve such as occurs in sprue. Bone marrow aspiration showed no change from the previous megaloblastic maturation arrest. On October 3, 1948, the patient was given 30 units of refined liver extract, Lederle, an amount which is roughly equivalent to the amount of vitamin B<sub>12</sub> injected previously. Again there was no reticulocyte response and the erythrocyte count continued to fall, so that by 10-13-48 ten days after the liver extract the erythrocytes numbered 840 000 per cu mm and the hemoglobin 4 Gm/100 cc. Bone marrow examination again revealed the same maturation arrest. Although the patient was unchanged clinically, he was given 500 cc of whole blood at this point in order to avoid the possibility that retransfusion might be necessary during the next therapeutic trial period.

On October 16, 1948, the patient was started on a ten day course of folic acid, 7.5 mg each day intramuscularly, so that the total dose of 75 mg would be roughly comparable to 30 units of refined liver extract. A reticulocyte response occurred and reached its maximum of 16.9 per cent on the fifth day. Clinical improvement was striking. The erythrocyte count increased slowly but by 10-29-48 when he was discharged it had risen to 2,410 000 cells per cu mm and the hemoglobin was 9.4 Gm per cent. Subsequently his blood counts have continued to rise toward normal. The bone marrow reverted to a normoblastic phase of maturation. The neurologic signs did not change appreciably.

During two days preceding folic acid therapy and the three days following the first dose of folic acid 24 hour urine samples were collected and their respective contents of folic acid measured through the courtesy of Dr. A. L. Franklin of Lederle Laboratories. The results were as follows:

Date	Urine Vol. ml	Therapy mg folic acid	Micrograms folic acid/ml	% excretion
10-8-48	750	—	0012	—
10-9-48	750	—	0015	—
10-17-48	800	7.5	3.0	32
10-18-48	425	7.5	6.0	33
10-19-48	750	7.5	5.0	49

These excretion values are within the range excreted by normal subjects.

A gastric analysis with histamine stimulation repeated in December 1948 revealed

Specimen	Free acid	Combined acid	Total acid
Pre histamine	13	5	18
Post histamine	19	11	30

### DISCUSSION

In 1936, Israels and Wilkinson<sup>1</sup> described a type of macrocytic anemia similar morphologically to pernicious anemia, but lacking the clinical manifestations of achylia gastrica, neural involvement, jaundice and glossitis. The bone marrow was megaloblastic, but the anemia responded poorly or not at all to the usual parenteral liver therapy. The authors suggested that achrestic anemia might be a suitable

name since they felt that liver extract was not utilized properly. In 1937, Wills, Clutterbuck and Evans<sup>2</sup> reported that experimentally induced macrocytic anemia in monkeys failed to respond to refined liver extracts such as Anahaemin but responded quite well to crude extracts such as Campolon. This was followed in 1938 by clinical reports by Napier<sup>3</sup> et al. and Wills and Evans<sup>4</sup> of cases of tropical macrocytic anemia which failed to respond to Anahaemin, but subsequently responded to Campolon. It was from this work that the term "Wills factor" appeared, to designate an unknown active factor in crude liver. Numerous reports of similar experience followed from other countries.<sup>5-7</sup>

In England, Davidson, Davis and Innes<sup>8</sup> in 1943, later Davidson and Girwood<sup>9</sup> in 1946 and Davidson<sup>10</sup> in 1948 described a total of 25 cases which they called "idiopathic refractory megaloblastic anemia." All were refractory to refined liver extract. Nine of these cases received in addition, iron, ascorbic acid and transfusions and improved slowly. Twelve patients received proteolyzed liver, a papain digest of whole liver administered orally, with a prompt response in 8 and a moderate response in the remaining 4. The daily dose of the proteolyzed liver was shown to contain only 0.4 mg. of folic acid. The authors therefore felt that there is still another unknown hematinic principle in the proteolyzed liver. The remaining 5 cases were given folic acid with a prompt, but submaximal response. They required proteolyzed liver to attain normal erythrocyte values. These authors also reported 34 other cases of refractory megaloblastic anemia which were associated with pregnancy, the puerperium or the sprue syndrome.

In 1946, Watson and Castle<sup>11</sup> reported 4 cases of nutritional macrocytic anemia which were refractory to parenteral liver therapy. These patients had in common an inadequate diet, free hydrochloric acid in the gastric contents, absent neural manifestations and normal lingual papillae. Bone marrow morphology was not reported. In 2 of the patients, the anemia occurred during pregnancy. The first 2 patients responded to liquid extract of liver (Valentine). The third responded to the oral administration of a suspension-solution of powdered liver extract (Lilly) and the fourth responded to the daily intravenous injection of 20 cc. of the supernatant of this special liver preparation. The fourth patient received 13 mg. of L. casei factor daily for 10 days along with other members of the vitamin B complex without benefit prior to the administration of liver extract. These authors concluded that the crude liver preparations given in large doses contained some hematoopoietic factor not present in the more refined liver extracts. They were willing to designate this substance the "Wills factor," and did not believe that it was folic acid.

Waldenström<sup>12</sup> in 1947 reported 4 cases of refractory macrocytic anemia, 3 of which responded to folic acid given by mouth. Two of these had responded previously to liver and then had become refractory. The one case that did not respond to folic acid was classed as idiopathic steatorrhea.

Recently Bethell and co-workers<sup>13</sup> reported briefly a case of puerperal macrocytic anemia in a 19 year old mother who had a megaloblastic marrow, free acid in the gastric juice, and glossitis. This anemia did not respond to a total of 10 gamma

of  $B_{12}$  intramuscularly over a ten day period, but responded to 10 mg of folic acid by mouth per day

The patient described in our report differs in certain minor clinical respects from most of the other cases of liver extract refractory macrocytic anemia. Objective neurologic signs were present which were probably manifestations of mild peripheral neuritis and cerebral atrophy induced by alcohol. The patient also had an acute glossitis which was unrelieved by vitamin  $B_{12}$  and refined liver extract, but responded to folic acid. In these respects he was similar to several patients with extrinsic factor deficiency reported by Moore, Vilter, Minnich and Spies.<sup>14</sup> However, failure to respond to purified liver extract makes such an etiology untenable. Pernicious anemia is eliminated by the return of free hydrochloric acid after treatment, and sprue seems unlikely without evidence of diarrhea or steatorrhea. There had been no gastro-enteric surgery and his gastro-enteric tract was normal when visualized with barium. By elimination he must be classified as liver extract refractory macrocytic anemia due to unknown causes. The hematopoietic effect of folic acid is similar to results reported by European clinics.

It seems probable that persons said to have achrestic anemia, Wills factor deficiency anemia and many instances of megaloblastic anemia of infancy and pernicious anemia of pregnancy, are all due to the same fundamental chemical deficiency.

The evidence in our case fails to demonstrate a primary etiologic role for folic acid deficiency because the excretion of this substance after parenteral injection was within the range expected in normal persons. For this same reason, a defect in the folic acid conjugase system is unlikely. Therefore, one must assume the existence of another factor, which acts in conjunction with folic acid in the process of erythrocyte maturation. The identity of this factor is unknown but it must be present in crude liver preparations such as proteolyzed liver. If this is true, folic acid in relatively large doses can overcome a deficiency of this factor by a mass action effect, a mechanism which may also explain its hematopoietic action in pernicious anemia where it overcomes a chemical deficiency, probably of vitamin  $B_{12}$ , conditioned by lack of intrinsic factor in the gastric juice.

Studies on the growth requirements of bacteria link folic acid<sup>15</sup> to thymine and vitamin  $B_{12}$ <sup>16</sup> to thymidine synthesis and suggest that these factors are intimately related to purine and pyrimidine metabolism. The inhibition of folic acid antagonists on bacterial growth and the ability of purines and pyrimidines to overcome or circumvent the inhibition<sup>17</sup> demonstrate these interrelationships also. The hematopoietic effect of large amounts of a pyrimidine, thymine, in human pernicious anemia, nutritional macrocytic anemia and sprue suggests that these concepts may be applicable to human nutrition.<sup>18</sup> The possibility that one of the points of breakdown of hematopoiesis in pernicious anemia may be the failure to convert thymine to thymidine has been suggested before.<sup>19</sup> It is likely that chemical chain reactions leading to the formation of nucleo-protein from amino acids are catalyzed by these factors derived from liver, and that folic acid and the unknown factor are essential for one step and vitamin  $B_{12}$  for a closely related one. Under such circumstances any one of these factors given in large doses, could overcome temporarily defi-

ciencies of the others, and thymine, one of the substrates of the reaction, would be effective in very large doses. Such a theory offers an explanation for the effect of folic acid in the patient described in this report and helps to explain many puzzling problems which have arisen in the field of macrocytic anemias.

### SUMMARY AND CONCLUSIONS

1 The patient described in this report had macrocytic anemia, megaloblastic maturation arrest in the bone marrow, glossitis, hyper-reflexia and diminished vibration perception in the feet. None of these abnormalities was improved by liver extract or vitamin B<sub>12</sub> but all responded rapidly to folic acid except the neurologic signs.

2 This patient appears to have had a megaloblastic anemia which has been described in European clinics under the names achrestic anemia and refractory megaloblastic anemia. It appears to be similar to Wills factor deficiency anemia and some cases of pernicious anemia of pregnancy.

3 This patient did not appear to have a primary deficiency of folic acid since the excretion of this substance in the urine was within normal limits. A deficiency of an unknown factor probably equivalent to the Wills factor is suggested.

4 It seems likely that folic acid induced a remission in this case by a mass action effect. The possible relationship of folic acid, vitamin B<sub>12</sub>, the unknown factor and liver extract to nucleo-protein synthesis is discussed.

### ADDENDUM

Since the completion of this paper, the patient herein reported, has been readmitted in hematologic relapse. He had received no interim treatment due to his failure to report back to us. During his second stay in the hospital he was treated with thymine, 13.2 Gm daily for ten days. Reticulocytosis of 10 per cent occurred and a rise in erythrocytes and hemoglobin is in progress. This hematologic response is consistent with the theory outlined above.

### ACKNOWLEDGMENT

We wish to thank Doctor Charles Foertmeyer for referring this patient to us for the clinical study used in this report.

### REFERENCES

- <sup>1</sup> ISRAELS M. C. G. AND WILKINSON J. F. Achrestic anemia. *Quart J Med* 5: 69, 1936.
- <sup>2</sup> WILLS L., CLUTTERBUCK P. W. AND EVANS B. D. F. New factor in production and cure of certain macrocytic anemias. *Lancet* 1: 311, 1937.
- <sup>3</sup> NAPIER L. E., DAS GUPTA C. R., CHAUDHURI R. N., SEN G. N., RAI CHAUDHURI M. N., SEN GUPTA P. C. AND MAJUMDER D. N. Anahaemin in tropical macrocytic anemia. *Indian Med Gaz* 73: 385, 1938.
- <sup>4</sup> WILLS L. AND EVANS B. D. F. Tropical macrocytic anemia. Its relationship to pernicious anemia. *Lancet* 2: 416, 1938.
- <sup>5</sup> UNOLEY C. C. Some deficiencies of nutrition and their relation to disease. *Lancet* 1: 915, 1938.
- <sup>6</sup> FAIRLEY N. H. Tropical macrocytic anemia in Indians treated with Anahaemin. *Lancet* 1: 1118, 1940.
- <sup>7</sup> RODRIGUEZ MOLINA R. Hematology of sprue. Report on 100 cases in Puerto Rico. *Puerto Rico J Pub Health & Trop Med* 15: 89, 1939.

- <sup>8</sup> DAVIDSON, L. S. P. DAVIS L. J., AND INNES J. Studies in refractory anemia. Technique and interpretation of sternal puncture biopsies. Classification. Edinburgh M. J. 50: 226, 1943.
- <sup>9</sup> — AND GIRWOOD, R. H. Folic acid in treatment of megaloblastic anemia. Lancet 2: 373, 1946.
- <sup>10</sup> — Refractory megaloblastic anemia. Blood 3: 107, 1948.
- <sup>11</sup> WATSON, J., AND CASTLE, W. B. Nutritional macrocytic anemia especially in pregnancy. Am J M Sc 211: 513, 1946.
- <sup>12</sup> WALDENSTRÖM, J. Successful treatment of liver refractory anemia with synthetic *Lactobacillus casei* factor. Blood 2: 426, 1947.
- <sup>13</sup> BETHELL, F. H. METERS, M. C. AND NELIGH, R. B. Vitamin B<sub>12</sub> in pernicious anemia and puerperal macrocytic anemia. J Lab & Clin Med 33: 1477, 1948.
- <sup>14</sup> MOORE, C. V. VILTER, R. MINNICH, V., AND SPIES, T. D. Nutritional macrocytic anemia in patients with pellagra or deficiency of the vitamin B complex. J Lab & Clin Med 29: 1226, 1944.
- <sup>15</sup> STOKES, J. L. Substitution of thymine for folic acid in the nutrition of lactic acid bacteria. J Bact 48: 201, 1944.
- <sup>16</sup> SHIVE, W., RANEL, J. M., AND HARDING, W. M. An interrelationship of purines and vitamin B<sub>12</sub>. J Biol Chem 176: 991, 1948.
- <sup>17</sup> RODGERS, L. L. AND SHIVE, W. Biochemical transformations as determined by competitive analogue. Metabolite growth inhibitions. J Biol Chem 172: 751, 1948.
- <sup>18</sup> FROMMETER, W. B. JR. SPIES, T. D. VILTER, C. F., AND ENGLISH, A. Further observations on the antianemic properties of 5-methyl uracil. J Lab & Clin Med 31: 643, 1946.
- <sup>19</sup> Vitamin B<sub>12</sub>, folic acid and thymidine. Nutr Rev 7: 19, 1949.



## A NOTE ON THE EFFECTIVENESS OF VITAMIN B<sub>12</sub> IN THE TREATMENT OF TROPICAL SPRUE IN RELAPSE

By RAMON M. SUAREZ, M.D., TOM D. SPIES, M.D., F. HERNANDEZ MORALES, M.D., AND ENRIQUE PEREZ, M.D.

*With the technical assistance of Miss CLEMENCIA BENITEZ-GAUTIER*

A SHORT time ago, vitamin B<sub>12</sub> was isolated<sup>1-5</sup> and shown to have a profound effect on blood regeneration in persons with pernicious anemia, nutritional macrocytic anemia, tropical sprue and nontropical sprue.<sup>6-9</sup> It also was found to be beneficial in relieving the acute and subacute combined degeneration of the spinal cord which so often is associated with pernicious anemia.<sup>10-13</sup> However, until very recently the amounts of vitamin B<sub>12</sub> available have been so small that investigators have not had sufficient amounts to treat patients fully. We decided to use part of our small supply of this material to make an intensive study of 3 patients with tropical sprue and to treat them over a considerable period of time, the thought being that it would probably take much larger amounts of vitamin B<sub>12</sub> to produce full remission than might be apparent from the dramatic hemopoietic response produced by minute doses. These 3 patients, studied in the hospital under controlled conditions, indicate that such is the case. The three following case histories of these patients illustrate their clinical and hemopoietic response to vitamin B<sub>12</sub> administered at fairly frequent intervals over a period of from 138 to 160 days.

These patients were selected for study by the following criteria: (1) The patient must have macrocytic anemia as determined by Wintrobe indices. (2) The bone marrow must show the typical megaloblastic type of maturation arrest seen in macrocytic deficiency anemias. (3) The erythrocyte counts must be below 2.5 million. (4) The patient must be untreated, or must not have been treated recently enough to interfere in any way with the evaluation of vitamin B<sub>12</sub> as a therapeutic agent. (5) He must have persistently low reticulocyte counts during the preliminary period of observation. (6) He must have alimentary tract symptoms consistent with the diagnosis of tropical sprue.

Pipets certified by the United States Bureau of Standards were used for the red cell counts. The hemoglobin content was determined by means of the Photovolt photoelectric hemoglobinometer, calibrated so that 14.5 grams was equivalent to 100 per cent. The reticulocytes were counted in dry preparations of brilliant cresyl blue counterstained with Wright's stain. Platelets were enumerated in the counting chamber used for red blood cells by means of a fresh solution of sodium citrate.

Sternal bone marrow was obtained by aspiration prior to treatment and again near the peak of reticulocytosis.

From the School of Tropical Medicine of Puerto Rico and Northwestern University Department of Nutrition and Metabolism.

This study was aided by a grant from the Martha Leland Sherwin Fund. The vitamin B<sub>12</sub> used in this study was supplied by Dr. Augustus Gibson, Merck and Company, Rahway, New Jersey.

Gastric analyses were performed in each case

On admission the patients were given the preliminary sprue diet previously described<sup>14</sup> and were maintained on this diet throughout the period of study. After the baseline studies were completed, the three patients selected were treated with vitamin B<sub>12</sub> at intervals of from 138 to 160 days

*Case 1* D.G., a 28 year old Puerto Rican woman was admitted to the hospital in May 1948 complaining of loss of appetite, soreness of the tongue and diarrhea characterized by frequent light-colored foamy stools

Family history and past history Irrelevant

Present illness The patient was in good health until after the birth of a normal child four years prior to her admission. At this time she lost her appetite, had occasional nausea and vomiting and developed diarrhea consisting of from six to eight soft, bulky, foamy, foul smelling, light yellow stools daily. During the following eight months she grew progressively weaker and lost 17 pounds in weight. At the end of this time she came to the Out-Patient Department of the hospital where a diagnosis of tropical sprue was made. She was given 8 cc. of crude liver extract three times a week. Following this therapy she improved only slightly and then very slowly. She became discouraged and stopped coming for treatment. By April 1948 she again developed loss of appetite, soreness of the tongue and severe foamy diarrhea. Within a month she was so weak she came to the hospital and was admitted for treatment.

Physical examination showed a poorly-developed, undernourished young woman who was obviously and chronically ill. The mucous membranes were very pale. The tongue was smooth and red, especially at the tip and edges.

Gastric analysis showed free hydrochloric acid in the gastric contents. The initial blood values were: red blood cells 2.41 million, hemoglobin 7.6 grams (48 per cent), reticulocytes 1.0 per cent, as can be seen in figure 1. She was given a total of 210 micrograms of vitamin B<sub>12</sub> in nine injections in a period of 147 days. Fifteen days after the last injection her blood values were: red blood cells 4.12 million, hemoglobin 10.1 grams (71 per cent), reticulocytes 0.8 per cent. The details of the hematologic response are shown in figure 1.

There was gradual clinical improvement. The soreness of the tongue and the diarrhea disappeared. When she was discharged after 166 days in the hospital she had gained 27½ pounds in weight and felt able to work.

*Case 2* E.R., a 54 year old Puerto Rican woman was admitted to the hospital in May 1948 complaining of progressive weakness, burning and soreness of the tongue and numbness of the extremities.

Family history and past history Irrelevant

Present illness Four years prior to this admission to the hospital her illness began insidiously with general debility and difficulty in walking. One and a half years later she developed soreness of the tongue and diarrhea consisting of liquid, foamy stools, light yellow in color. Following treatment with liver extract the diarrhea improved, the burning of her tongue disappeared and she gained in strength. She continued liver therapy for six months, then for economic reasons discontinued it. A few months later she again developed general debility and numbness of the legs, but no diarrhea. She was admitted to the hospital where a diagnosis of sprue was made. Following treatment with liver extract she improved clinically and hematologically and was discharged from the hospital forty-five days after admission. She failed to return for further treatment and one year later she was admitted to the hospital again, complaining of progressive weakness, soreness of the tongue and numbness of the lower extremities, but no diarrhea.

Physical examination showed a pale woman in no acute distress but obviously weak and chronically ill. The skin and mucous membranes were pale. The sclera had a slight icteric tint. The tongue was red at the tip and edges. The vibratory sense was intact.

Gastric analyses showed free hydrochloric acid in the gastric juice. Her initial blood values were: red blood cells 1.59 million, hemoglobin 5.0 grams (32 per cent), reticulocytes 0.1 per cent, as can be seen in figure 2. She was given a total of 205 micrograms of vitamin B<sub>12</sub> in nine injections in a period of 166 days. Twelve days after the last injection her blood values were: red blood cells 4.21 million, hemoglobin 9.7



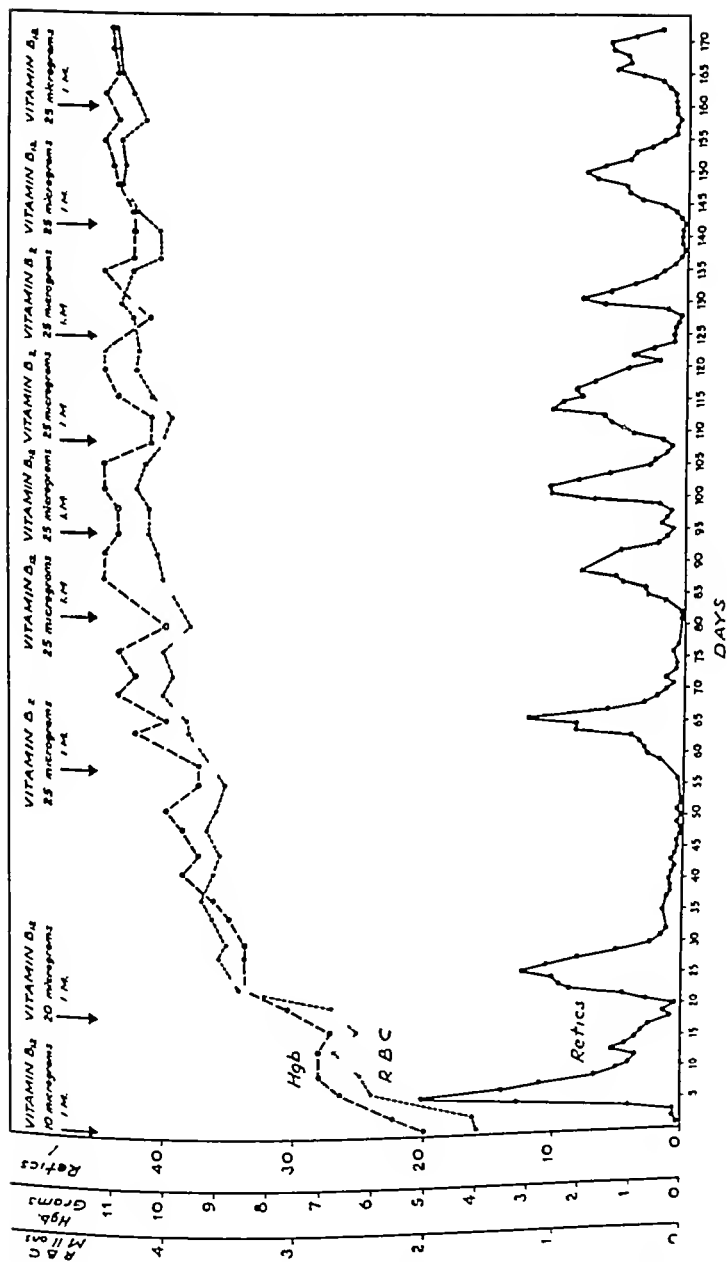
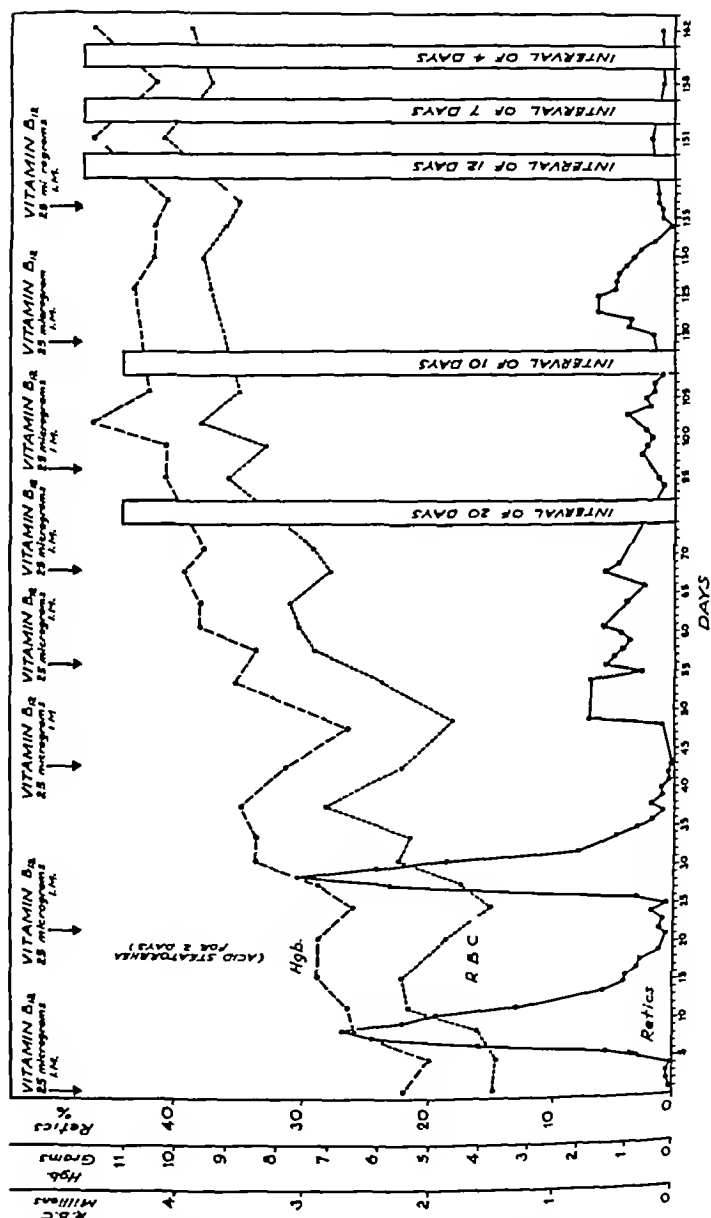


FIG. 1.—Hemopoietic response of E. R. a patient with tropical sprue to vitamin B<sub>12</sub>.

FIG 3—Hemopoietic response of J. G. a patient with tropical sprue to vitamin B<sub>12</sub>

11.1 grams (72 per cent), reticulocytes 18 per cent. The details of her hematologic response can be seen in figure 2.

She had a striking clinical improvement and was discharged from the hospital 185 days after admission. Since then she has been seen several times and is doing her work and has remained well. Some numbness of both legs persists. The vibratory sense remained intact.

**Case 3 J G**—a 73 year old Puerto Rican woman was admitted to the hospital in June 1948 complaining of foamy diarrhea, burning of the tongue and general weakness.

**Family history**—One sister died probably of sprue or pernicious anemia. **Past history**—Irrelevant.

**Present illness**—She was well until six months prior to her admission when she lost her appetite, developed foamy diarrhea and soreness of the tongue. She rapidly lost strength and during the six months she was ill lost 68 pounds in weight.

**Physical examination** showed a very ill pale woman. She had atrophic glossitis. The abdomen was flatulent and distended.

**Gastric analysis** showed free hydrochloric acid in the gastric juice. Her initial blood values were: red blood cells 1.49 million, hemoglobin 5.5 grams (35 per cent), reticulocytes 0.2 per cent, as can be seen in figure 3. She was given a total of 200 micrograms of vitamin B<sub>12</sub> in eight injections in a period of 138 days. There was definite improvement in her stools. Twenty-four days after the last injection her blood values were: red blood cells 3.89 million, hemoglobin 11.6 grams (75 per cent), reticulocytes 1.2 per cent. The details of her hematologic response can be seen in figure 3.

#### COMMENT

The three patients with tropical sprue reported were repeatedly given injections of crystalline vitamin B<sub>12</sub> intramuscularly. Case 1 was given a total of 210 micrograms in nine injections ranging in amounts from 10 to 25 micrograms in a period of 147 days. Case 2 received a total of 205 micrograms in nine injections ranging in amounts from 10 to 25 micrograms in a period of 160 days. Case 3 was given a total of 200 micrograms in eight 25 microgram injections in a period of 138 days. In each case there was little or no detectable change for the first three or four days, then, when the reticulocytes began to rise in the peripheral blood on the fourth or fifth day, the patients began to feel better. Following the reticulocyte peak which occurred from the sixth to the ninth day the red blood cells and hemoglobin gradually increased. In each case there was gradual gain in strength, and in patients 1 and 2 who had diarrhea there was some improvement in their alimentary tract function although the stools did not become entirely normal.

No final conclusions as to dosage and intervals between injections can yet be made but no therapeutic agent thus far used in the treatment of tropical sprue has been so effective per unit of weight as vitamin B<sub>12</sub>.

#### SUMMARY

Three cases of tropical sprue were treated with repeated injections of vitamin B<sub>12</sub> and showed dramatic and sustained therapeutic responses.

#### REFERENCES

- <sup>1</sup> SHORR M. S. Activity of vitamin B<sub>12</sub> for the growth of *Lactobacillus lactis*. *Science* 107: 397, 1948.
- <sup>2</sup> RICKES, E. L., BRINK N. G., KONIUSZY F. R., WOOD T. R., AND FOLKERS K. Crystalline vitamin B<sub>12</sub>. *Science* 107: 396, 1948.
- <sup>3</sup> SMITH E. L. Purification of anti-pernicious anemia factors from liver. *Nature* 161: 638, 1948.
- <sup>4</sup> RICKES E. L., BRINK N. G., KONIUSZY F. R., WOOD T. R., AND FOLKERS K. Vitamin B<sub>12</sub> and its complex. *Science* 107: 134, 1948.

- <sup>6</sup> SMITH E L. Presence of cobalt in the anti pernicious anaemic factor *Nature* 162, 144, 1948
- <sup>7</sup> WEST RANDOLPH. Activity of vitamin B<sub>1</sub> in Addisonian pernicious anemia. *Science* 107, 398, 1948
- <sup>8</sup> SPIES T D, STONE R E AND ARAMBURU T. Observations on the anti anemic properties of vitamin B<sub>12</sub>. *South M J* 41, 522, 1948
- <sup>9</sup> — GARCIA LOPEZ G, MILANES F, LOPEZ TOCA R AND CULVER B. Observations on the hemopoietic response of persons with tropical sprue to vitamin B<sub>12</sub>. *South M J* 41, 523, 1948
- <sup>10</sup> — SUAREZ R M, GARCIA LOPEZ G, MILANES F, STONE R E, LOPEZ TOCA R, ARAMBURU T, AND KARTUS S. Tentative appraisal of vitamin B<sub>1</sub> as a therapeutic agent. *J.A.M.A.* 139, 521, 1949
- <sup>11</sup> STONE R E AND SPIES, T D. Vitamin B<sub>12</sub> and subacute combined degeneration of the spinal cord. *Tirage a part de la Revue Internationale de Vitaminologie* Tome XX, Cahier 1/3, 1948
- <sup>12</sup> SPIES T D, STONE R E, KARTUS S AND ARAMBURU T. The treatment of subacute combined degeneration of the spinal cord with vitamin B<sub>1</sub>. *South M J* 41, 7030, 1948
- <sup>13</sup> BERE L, DENNY BROWN D, FINLAND M AND CASTLE W B. Effectiveness of vitamin B<sub>12</sub> in combined system disease. *New England J M* 239, 328, 1948
- <sup>14</sup> — CASTLE W B, WELCH A D, HEINLE R W, ANKER R. AND EPSTEIN M. Observations on the etiologic relationship of achylia gastrica to pernicious anemia. X. Activity of vitamin B<sub>12</sub> as food (extrinsic) factor. *New England J M* 239, 911, 1948
- <sup>15</sup> SUAREZ R M, SPIES T D, AND SUAREZ R M, JR. The use of folic acid in sprue. *Ann. Int. Med.* 26, 643, 1947

# DICUMAROL THERAPY CONTROLLED BY THE STABILIZED THROMBIN METHOD FOR DETERMINATION OF PROTHROMBIN

By L. A. STERNBERGER, M.D.

**A**N ADEQUATE method of control for the anticoagulant effect of dicumarol is an essential condition for the evaluation of effectiveness and danger of this therapy. Such a method should control the action of dicumarol only, it should be independent of accidental variations in clotting factors due to other causes. As long as these requirements are not fulfilled, the optimal dosage of dicumarol cannot be determined with too large doses hemorrhage results, while too small doses make it impossible to obtain the full therapeutic effect of the drug.

Hitherto the one-stage method of Quick<sup>1</sup> or modifications of it have been used exclusively in clinical work. It rests upon the principle that if in the coagulation of blood plasma thromboplastin, calcium and fibrinogen concentration are kept constant the clotting time depends only on prothrombin provided that these four factors are the only coagulation factors existing. Thromboplastin is controlled by addition of excess of this factor. The activity of thromboplastin has to be determined by standardization with a relatively large number of normal control plasmas.<sup>2</sup> Since the normal controls are to be used for standardization of a reagent for the method, the absolute value of prothrombin in normals cannot be established. Calcium concentration is to remain at its constant optimum. But it was pointed out by Jacques and Dunlop<sup>3</sup> and by Quick<sup>4</sup> that the prothrombin time of hypoprothrombinemic oxalated plasma is very sensitive to changes in calcium concentration so that in such plasmas this requirement becomes very hard to fulfill. The prothrombin clotting time of hypoprothrombinemic undiluted plasma is compared with that of diluted normal plasma. Dilution with normal saline changes the fibrinogen concentration significantly and although Niesche and co-workers<sup>5</sup> have attempted to avoid this by using fibrinogen as diluent and Rosenfield and Tuft<sup>6</sup> by employing deprothrombinized plasma, the results have not been uniform.<sup>7</sup>

The one-stage method does not take into account any of the following coagulation factors, which are capable of influencing the prothrombin time: (1) Antithrombotic substances described by Astrup<sup>7</sup> and by Glazko and Ferguson<sup>8</sup> which destroy thrombin immediately after its formation. (2) Owren's fifth coagulation factor<sup>9</sup> which is required in addition to thromboplastin and calcium to convert prothrombin to thrombin, and variations of which may increase or decrease the prothrombin time. (3) Autocatalytic factors described by Astrup<sup>7</sup> and Owren.<sup>9</sup> Because of such factors the rate of conversion of prothrombin to thrombin is not constant. The principle of the one stage method is that the rate of conversion of prothrombin to thrombin is dependent on the concentration of prothrombin by a definite relationship. (4) Inhibition factors postulated by Ferguson and Glazko<sup>10</sup> and by Tocantins<sup>11</sup> which slow down the conversion rate of prothrombin to thrombin. Moreover, normal plasma may contain any number of unknown factors<sup>12</sup> which affect the thromboplastin used in the one stage method so that, as pointed out by Conley and Morse<sup>11</sup> results obtained with different thromboplastins are not comparable. In fact, serious doubts arise as to whether the one-stage method gives more than a rough estimate of plasma prothrombin. On the other hand, the evaluation of the effectiveness and safety of dicumarol therapy can be made only if a strictly reliable control method is available and practical, giving absolute results so that reports from one laboratory can be compared with those obtained in another.

The stabilized thrombin two-stage method<sup>12</sup> is independent of any of the above factors of inaccuracy and, in addition, gives results in absolute units. With the use

From the Department of Pathological Physiology, Hebrew University and the Pichard-Hadassah University Hospital, Jerusalem, Israel.



of the modification to be described in this paper (related particularly to the preparation of a stable thrombin reagent and to different quantities of materials used in the test) it does not take more time of performance than the one stage procedure. The method is based on our observation that alcohol suppresses the antithrombic activity of plasma, so that it becomes possible to keep constant the amount of thrombin obtained after quantitatively converting prothrombin to thrombin.

### METHOD FOR DETERMINATION OF PROTHROMBIN

#### *Principle*

The oxalated plasma used contains the prothrombin to be examined, as well as antithrombin and fibrinogen. First, the fibrinogen is removed by adding thrombin. Fibrinogen becomes thus converted to fibrin, and is rolled out with a stirring rod. The resulting fluid contains all the prothrombin, antithrombin, and the added thrombin (but not fibrinogen). However, within ten minutes the added thrombin will have been inactivated by the antithrombin present. Now the antithrombin activity will be suppressed by the addition of alcohol, and prothrombin converted to thrombin with human milk (thromboplastin) and calcium. The resulting fluid contains all the thrombin obtained quantitatively from the prothrombin originally present (but does not contain active antithrombin). It does not clot as such, because fibrinogen has been removed previously. The amount of prothrombin is now determined by adding various dilutions of the thrombin thus obtained to constant amounts of normal plasma and recording the thrombin-fibrinogen clotting times. This method is independent of the activity of the reagents used. Thus, prothrombin concentration can be directly read from the thrombin-fibrinogen clotting times, and no comparison with normal controls is necessary.

#### *Reagents*

- 25 per cent by volume of ethyl alcohol in normal saline solution
- 50 per cent by volume of ethyl alcohol in normal saline solution
- 0.1 M sodium oxalate solution
- 0.2 M calcium chloride solution
- Thrombin solution
- Fresh normal oxalate plasma
- Human milk

#### *Preparation of Thrombin Solution<sup>12</sup>*

A temperature between 16 and 22 C is maintained while the following ingredients are placed successively into a flask and stirred after each addition:

- 380 parts of 50 per cent by volume of ethyl alcohol in normal saline solution
- 145 parts of normal saline solution
- 25 parts of 0.2 M calcium chloride solution
- 210 parts of human blood (whole blood - 9 parts of blood obtained by venepuncture and rendered in coagulable by addition to 1 part of 0.1 M sodium oxalate solution)
- 75 parts of human milk
- 75 parts of 50 per cent by volume of ethyl alcohol in normal saline solution

The material obtained after the lapse of about 5 to 10 minutes (crude thrombin) will clot an equal volume of human plasma in 6 to 8 seconds. It is very stable. It may be processed immediately or may be kept in the refrigerator without loss of activity for at least eight months.

To 32 parts of crude thrombin (shaken well to obtain a uniform suspension) are added 18 parts of

95 per cent ethyl alcohol by volume. The whole is shaken violently and centrifuged immediately in an angle centrifuge. The sediment obtained is resuspended in 16 parts of oxalated merthiolate saline solution (prepared by placing 2 parts of 0.1 M sodium oxalate solution and 10 parts of 1 per cent merthiolate [sodium ethyl mercurithiosalicylate] into a volumetric flask and making up to 100 with normal saline solution). The suspension is stirred to break up the sediment as completely as possible, whereafter it is centrifuged and the sediment discarded. The supernatant thrombin solution will clot an equal volume of fresh oxalate plasma in 4 seconds. It is stable for at least six months storage in the ice box.

*Fresh human plasma.* Nine ml. of blood are drawn by venepuncture from a normal subject with as little trauma as possible and added immediately to 1 ml. of 0.1 M sodium oxalate solution contained in a centrifuge tube. The plasma is obtained by centrifugation.

*Human milk.* It may be used fresh or it may be stored in the ice box for at least one month. If milk has been stored it should be well shaken to obtain a uniform suspension.

#### *Procedure for the Determination of Prothrombin*

Four and five-tenths ml. of blood are drawn by venepuncture and added as rapidly as possible to a centrifuge tube containing 0.5 ml. of 0.1 M sodium oxalate solution. The tube is shaken immediately by inverting it three times. The plasma is obtained by centrifugation.

The procedure to follow is done at a temperature range between 16 and 22 degrees centigrade.

*Step one, defibrination.* 0.5 ml. of thrombin are added to 1.0 ml. of the plasma. After 10 minutes the liquid is expressed from the clot by wrapping the latter around a glass rod (using best a pipet with a broken, rough end).

*Step two, thrombinization.* To 0.75 ml. of defibrinated plasma there are added successively

1.75 ml. of 25 per cent by volume of ethyl alcohol in normal saline solution

1.25 ml. of 50 per cent by volume of ethyl alcohol in normal saline solution

0.3 ml. of human milk

0.075 ml. of 0.2 M calcium chloride solution,

shaking after each alcohol addition and after the addition of the calcium.

*Step three, dilution.* Serial dilutions of the thrombinized, stabilized plasma obtained in step two should be set up not earlier than 10 minutes after thrombinization, and preferably not later than  $1\frac{1}{2}$  hours thereafter. If stored, rather than fresh milk is used for thrombinization, dilutions should be set up only after the lapse of 20 minutes after thrombinization. For dilution, a 25 per cent (by volume) solution of ethyl alcohol in normal saline is used.

*Step four, clotting.* With a pipet graduated to the tip, 0.2 ml. of various dilutions of thrombinized plasma are drawn into Wassermann tubes containing 0.2 ml. of fresh human plasma. At the moment of contact with the plasma a stop-watch is started. The tube is held—after brief shaking—against a screened source of light (an electric bulb screened by placing filter paper in front of it proves satisfactory). The test tube is tilted in a way that the fluid contained in it is allowed to flow in turn along its walls from the bottom of the tube towards its top, and back to the bottom again, and the moment of appearance of granularity is recorded by stopping the stop-watch. (The fibrin appears in the form of granules, rather than of threads because of the presence of alcohol. Therefore, the tube should not be rotated but tilted. End points are very sharp, if this procedure is followed.)

*Evaluation of the Amount of Prothrombin by the Stabilized Thrombin Method*

Results with this method are obtained in absolute values, and no comparison with a normal standard is necessary. In order to obtain comparable results with the one-stage method, we fix the normal value of prothrombin arbitrarily as 100. This corresponds (see table 1) to a clotting time of 18 seconds obtained when adding to 0.2 ml. of normal plasma 0.2 ml. of a 1:10 dilution of thrombinized plasma (i.e., a 1:80 dilution of the original plasma). In hypoprothrombinemic plasma this value will be obtained at a correspondingly lower dilution.

We usually set up dilutions of 40, 20, and 10 per cent for samples presumably normoprothrombinemic, and dilutions of 80, 40, and 20 per cent for hypoprothrombinemic thrombinized plasmas. Occasionally also dilutions of 60 and 30 per cent are set up, particularly in cases in which the clotting time of 18 seconds seems to occur in between 80 and 40 per cent. If the values recorded as normal in table 1 do not coincide exactly with any one of the dilutions actually set up, but happen to lie in between them, the significance of this can be evaluated by comparing the

TABLE 1 *Clotting Times of Various Dilutions of Thrombinized Stabilized Plasma*

Dilution of thrombinized plasma	Corresponding dilution of original plasma	Clotting time
%	%	seconds
100	12.5	6.2
80	10.0	6.9
60	7.5	7.8
40	5.0	9.2
20	2.5	12.6
10	1.25	18.0
5	0.625	24.7

values obtained at the various dilutions tested and observing whether corresponding deviations of the clotting times occur at all these dilutions. With this procedure the following values of prothrombin can be determined with accuracy, (more definite recording would be within the limits of experimental error) 200, 150, 100, 75, 50, 35, 25, 20, 17, 14, 12.5, 11, 10, 8.5, 7, 6, 5.5 and 5. Standard error and standard deviation of the clotting times recorded in table 1 have been computed in a previous publication.<sup>12</sup>

## DICUMAROL TREATMENT

In this series, 43 cases were given dicumarol for a period of seven to forty-five days. These include 27 cases of pulmonary embolism, 7 cases of thrombophlebitis, 4 cases of arterial embolism, and 5 postoperative cases treated prophylactically. All cases of arterial embolism and the more severe cases of pulmonary embolism were also given heparin for the first one or two days of treatment, usually until the prothrombin reached a level of 50. Heparin was always given by continuous intravenous drop infusion, and the venous blood coagulation time kept between 15

and 21 minutes. It is noteworthy, that the determination of prothrombin with our method is not influenced by the amount of heparin in the blood, since it excludes the effect of antithrombin and is not dependent upon the conversion time of prothrombin to thrombin. Thus, unlike with the one-stage method,<sup>14</sup> continuous administration of heparin does not disturb the determination of prothrombin.

#### *The Prothrombin Level before Treatment*

Frequently patients showed a hyperprothrombinemia of 150 to 200 before treatment. This was particularly marked in patients with long standing thrombosis before institution of treatment, or in cases of pulmonary embolism, especially in recurrent pulmonary embolism. On the other hand, among 78 postoperative determinations of prothrombin, there were also 11 cases of hyperprothrombinemia, yet none of them did develop postoperative thrombosis or embolism. It is our impression that the presence of hyperprothrombinemia cannot be used to predict whether a patient is predisposed to thromboembolic disease. It seems, however, that some patients, if having already contracted a thrombosis, may, after a certain lapse of time, develop an occasional hyperprothrombinemia.

#### *Determination of Dosage*

In all our patients we endeavored to keep the level of prothrombin between 17 and 50. Dicumarol is a slow acting and very cumulative drug. In attempting to keep the patient at a certain maintenance level, it is necessary to determine the dose to be given on a certain day not only by the prothrombin level for that particular day, but also by the previous response of the patient. Such maintenance was accomplished by the following program of dosage:

First day Always 300 mg. are given

Second day In patients with thromboembolic disease, 200 mg. are given, in all prophylactic cases and in every weak or emaciated patient, 100 mg.

Third day If the prothrombin for that day is 100 or above, 200 mg. are given. If it is 75-100 mg. or less, no dicumarol is given.

Fourth and each subsequent day If the prothrombin is above 50, 200 mg. are given. If it is 50, and was on the preceding day above 50, 100 mg. are given. If it is 50 and was on the preceding day 50 or less, 200 mg. are given. If the prothrombin is 35, and was on the preceding day more than 35, no dicumarol is given. If it is 35 and was on the preceding day also 35, 100 mg. are given. If it is 35, and was on the preceding day less than 35, 200 mg. are given. If the prothrombin is less than 35, no dicumarol is given.

Using this program of administration of dicumarol in the stabilized thrombin method for the determination of prothrombin, it is easy to keep the prothrombin level between 17 and 50. During a total of 513 determinations of prothrombin in the hypoprothrombinemic maintenance period of dicumarol in this series, only in 12 determinations (2.3 per cent) was the prothrombin more than 50 and only in three instances (0.6 per cent) was it less than 17. Daily variations of prothrombin were smaller with this method of control than with the one stage method. This becomes obvious, if it is borne in mind that the one-stage method is dependent also

on other factors besides the quantity of prothrombin (as outlined above), while the stabilized thrombin method is a direct measure of the amount of prothrombin after it has been converted to thrombin

### Comparison with the One-Stage Method

In a number of cases we have been running parallel determinations with the one-stage method of Quick.<sup>1</sup> An example is given in figure 1. The dosage of dicumarol was determined by the results of the stabilized thrombin method. It will be seen that in the beginning of treatment prothrombin values fell off more rapidly and after stoppage of dicumarol returned more quickly to normal with the one-stage than with the stabilized thrombin method. This discrepancy is explained if consideration is

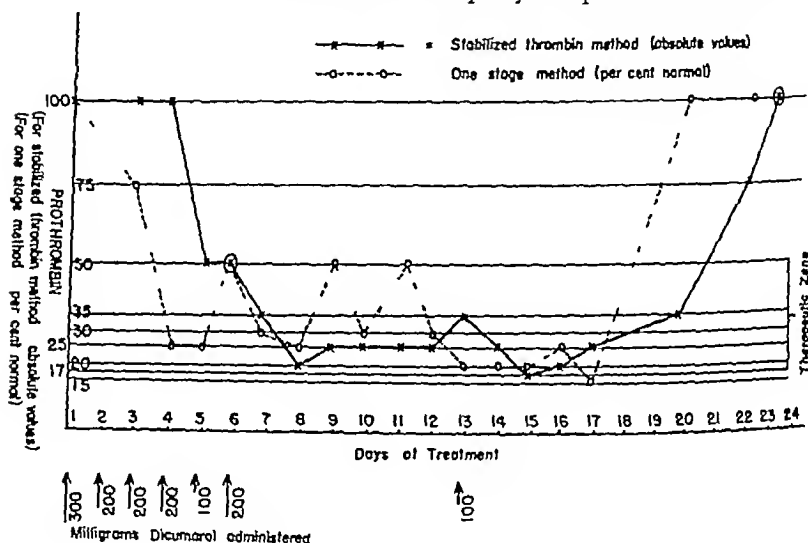


FIG. 1.—Course of Treatment in a Case of Bilateral Thrombophlebitis during Paratyphoid Fever (E. paratyphi B infection) Comparison of One Stage and Stabilized Thrombin Method

taken of the fact that the one-stage method is dependent on both, the quantity of prothrombin as well as the speed of conversion of prothrombin to thrombin, while the stabilized thrombin method is dependent only upon the quantity of prothrombin. It may well be that fresh circulating prothrombin is more active in its rapidity of conversion to thrombin than less recently formed prothrombin, while both, new and old prothrombin, still form the same amount of thrombin from a given amount of prothrombin. It was pointed out by Overman and co-workers<sup>18</sup> and by Witts<sup>19</sup> that dicumarol probably acts by inhibiting the formation of prothrombin in the liver through competition with vitamin K. Dicumarol hypoprothrombinemia is induced by slowing down the formation of new prothrombin. Therefore, in the beginning of the treatment, the relative amount of old prothrombin in circulation will predominate over that of newly formed prothrombin, and a lower value is obtained with the one-stage method than does correspond to the total amount of

prothrombin present. Upon stopping dicumarol new prothrombin is formed again, while the reserve of prothrombin in the circulating blood is relatively small. As a result the relative amount of newly formed prothrombin will predominate over that of old prothrombin, and a higher value of prothrombin is obtained with the one-stage method that does correspond to the quantity of prothrombin actually present. Similar results have been obtained by Hurn and Mann<sup>17</sup> in comparing the one-stage method with the two-stage method of Warner, Brinkhous, and Smith.<sup>18, 19</sup>

### *Treatment of Pulmonary Embolism*

Twenty-seven patients were treated. Thirteen of them had multiple emboli and were first seen only after a recurrent embolization. In none of these patients did dyspnea or cyanosis last for more than two days after the start of treatment, although 9 of the cases with recurrent pulmonary emboli were continually cyanotic and dyspneic from the time of occurrence of the first embolus till the second day after the start of treatment, i.e., for a period varying from 5 to 16 days (average 7 days). In only 1 of the 27 cases did a further infarction occur during treatment. It took place on the eighth day, and symptoms were mild. All patients recovered. They were allowed out of bed 3 to 7 days after all of the following conditions had been fulfilled: (1) absence of fever, (2) absence of blood in the sputum, (3) absence of large amounts of pleural exudate (but a patient was never kept in bed because of the presence of a few friction rales), (4) absence of continuous pain (few patients had still some slight pain on deep inspiration when first out of bed). Dicumarol therapy was continued for 1 to 2 days after the patient had been ambulatory. With this program the period of treatment in these 27 cases was minimum 5 days and maximum 30 days, average 16 days. The average daily dose of dicumarol was 121 mg. The time of recumbency of the patients had no relation to the initial severity of symptoms. The shortness of the period of morbidity is outstanding, particularly if it is considered that there was an exceptionally large proportion of severe cases in this series.

*Case Report.* A 54 year old man was admitted because of a repeated pulmonary embolus complicating thrombophlebitis. He was in a state of severe cyanosis and dulled consciousness. Respiration was of the Cheyne Stokes type; the pulse was too weak to be felt. Big rales could be heard from a distance. The patient had a generalized purpuric rash which was reported to have developed about three days before admission. The thrombocyte count was 180,000 per cu. mm.

The patient was given immediately 0.120 Gm. papaverine hydrochloride intravenously and was started upon a continuous intravenous drip infusion of heparin in glucose (100 mg. of heparin per 300 cc. of 5 per cent glucose solution). At the same time blood was withdrawn by venesection (300 ml.). Because of the severity of the condition the purpura was ignored and dicumarol was started. Heparin was discontinued after twenty-four hours of treatment.

The patient's condition improved rapidly. Twelve hours after the treatment had been started he began to respond to words; the cyanosis became less and the respiration regular. The pulse although still weak was improving.

After forty-eight hours the patient started to cough out a small amount of bloody, mammillary sputum; consciousness had fully returned; dyspnea had disappeared; cyanosis was fading. On the fourth day the patient wanted to get out of bed and on the fifth day there was no more blood in the sputum. On the eighth day the patient was allowed out of bed and on the ninth day treatment was ended.

In this case anticoagulant treatment was considered a matter of last resource and dicumarol was given, although the patient was having a purpuric rash on admission. Within twelve hours of treatment while heparin was being given the rosy purpuric spots became deep red in color. At this time the prothrombin was still 100. After interruption of heparin the purpura started to disappear while prothrombin was falling to be maintained at a level of 35. No erythrocytes were found in the urine at any time during the treatment.

### *Thrombophlebitis*

Seven cases, including 2 patients with thrombophlebitis during typhoid fever, were treated. In all but one patient did local pain disappear within three days of treatment. Some patients were seen first after having been suffering from the disease for weeks. In all patients there was a regression of the extent of local tenderness from the start of treatment on. In no case did pulmonary embolism develop. The period of treatment was minimum 4 days, maximum 45 days, average 19 days. The average daily dose of dicumarol was 119 mg.

### *Arterial Embolism*

There were only 4 cases of arterial embolism in this series. Three patients suffered from myocardial infarction and contracted emboli in the popliteal artery. They were given dicumarol and heparin (the latter was discontinued when the prothrombin level had reached 50) and in all of them the circulation had become restored within 4 days. All patients recovered from the myocardial infarction. One case of embolism of the retinal artery came to treatment only after vision had been lost for two days, and no improvement was observed during treatment.

### *Prophylactic Treatment*

Five cases were given dicumarol prophylactically after surgical intervention. Treatment was started on the second postoperative day and continued until the patient was out of bed. In none of these cases did thrombosis develop. The period of treatment was minimum 3 days, maximum 8 days, average 7 days. The average daily dose of dicumarol was 115 mg.

### *Relation of Dosage of Dicumarol to Clinical Condition*

The average daily dose of dicumarol was similar in all clinical conditions treated, although there were large individual variations. The most sensitive patient (pure prophylactic treatment) received a total of 400 mg during 8 days of treatment (average 50 mg daily), while the least sensitive patient (pulmonary embolism) received a total of 2400 mg during 12 days of treatment (average 200 mg daily).

### *Complications*

The only complication of dicumarol treatment is hemorrhage due to excessive hypoprothrombinemia. In this series there was only one case of bleeding (2 per cent). This was a wound hemorrhage in a cachectic patient receiving prophylactic treatment. As we desired, in this series, to establish the usefulness of our method of control we have never hesitated to give dicumarol to patients with relative contraindications to the use of anticoagulant therapy (see the case of purpura described

above) We believe that the hemorrhage did occur only because of disregard of such contraindications

*Case Report* A two-stage Lahey's abdominoperineal resection of a reticulosarcoma of the rectum was performed by Dr. Joseph on a 60 year old emaciated man. After the second operation he was rather dehydrated and was lying in bed listlessly without making any spontaneous movement. Prophylactic dicumarol treatment was instituted on the fourth postoperative day and continued until the eleventh postoperative day, as shown in the following table

Date	Prothrombin	Dicumarol (Gm.)
August 11	100	0.3
August 12		0.1
August 13	50	—
August 14	35	—
August 15	25	—
August 16	25	—
August 17	17	—
August 18	1 a.m. 12.5 9 a.m. 17	

The patient was very sensitive to small doses of dicumarol. The blood-tinged discharge from the perineal wound was present before and during the whole course of treatment, but the amount of blood did not increase until August 17. On this day prothrombin was still falling. Because of the anemia and general weakness the patient was given a transfusion of 800 ml. of banked blood. Fresh blood was not used because this transfusion was not given for hypoprothrombinemia. At 1 a.m. the following morning (August 18) 8 hours after this transfusion blood started to ooze from the operative wound. At this time prothrombin was 12.5. Immediately thrombin<sup>12</sup> was injected into the wound cavity and cessation of bleeding was instantaneous. The patient was also given Hykinone (menadiol bisulfite, Abbott) 0.072 Gm. by slow intravenous injection. Eight hours thereafter the prothrombin was 17 and bleeding did not recur. The patient made an uneventful recovery.

It may be possible that administration of banked blood diluted the patient's circulating prothrombin and thus initiated the bleeding. Also any of the following contraindications to the use of dicumarol may have predisposed to the bleeding: (1) an extensive, infected, operative wound,<sup>20</sup> (2) surgery of the gastro-intestinal tract,<sup>20</sup> (3) dehydration and cachexia.<sup>21</sup>

In all our patients repeated urinalyses for a search of erythrocytes were made. In none did microscopic hematuria appear during treatment, while in those patients who were treated after surgery of the urinary tract, hematuria never became more marked and often disappeared during dicumarol administration.

With the stabilized thrombin method it is possible to keep the patient well within the boundaries of the therapeutic zone and variations of prothrombin from day to day are relatively small. There were only three hyper-reactors (7 per cent) in this series, out of which only one (2 per cent) had a hemorrhage, and this would have been prevented, had we excluded cases with contraindications to dicumarol. With the use of the one-stage method 16.6 to 27 per cent of hyper-reactors are encountered,<sup>\*</sup> and hemorrhagic complications occur in 4.7 to 20 per cent, average 8.3 per cent.<sup>22</sup>



## CONCLUSIONS

The stabilized thrombin method for the determination of prothrombin is the only procedure which determines prothrombin quantitatively and is independent of other coagulation factors. Since it is independent of the activity of the reagents used, no normal controls are necessary.

In using the stabilized thrombin method for control of the clinical administration of dicumarol rather constant hypoprothrombinemic levels could be attained, and daily variations of prothrombin were relatively small. There were less hyperreactors and less hemorrhages than would be expected with the use of the one-stage method. Rarely did a patient's prothrombin rise above the therapeutic range during treatment.

The ease with which such safe and effective therapeutic levels can be maintained is explained by the fact that, while the one-stage method is dependent upon a number of coagulation factors, the stabilized thrombin method is a direct quantitative estimation of only prothrombin.

## ACKNOWLEDGMENTS

I am greatly indebted to Professor E. Wertheimer, Chief of the Department of Pathological Physiology, Hebrew University and Director of the Chemical Laboratory, Rothschild Hadassah University Hospital. His suggestions and encouragement have been an invaluable aid during this work.

The cooperation of Dr. Rachmilewitz, Dr. Kleeberg, Professor B. Zondek, Dr. Joseph and Dr. Nissel is also gratefully acknowledged.

## REFERENCES

- <sup>1</sup> QUICK, A. J. *The Hemorrhagic Diseases*. Springfield, Thomas, 1942.
- <sup>2</sup> AGOHLER, P. M., HOWARD, J., LUCIA, S. P., CLARK, W. AND ASTAFF, A. Standardization of the Quick prothrombin test. *Blood* 1: 220, 1946.
- <sup>3</sup> JACQUES, L. B. AND DUNLOP, A. P. The effect of calcium concentration on prothrombin time. *Am. J. Physiol.* 145: 67, 1945.
- <sup>4</sup> QUICK, A. J. On the quantitative relationship between calcium and prothrombin. *Am. J. Physiol.* 148: 211, 1947.
- <sup>5</sup> NITSCH, S. A., GERARDE, H. W. AND DEUTSCH, H. F. Prothrombin time studies on human beings and experimental animals. *J. Lab. & Clin. Med.* 32: 410, 1947.
- <sup>6</sup> ROSENFELD, R. E., AND TUFT, H. S. Estimation of prothrombin level from prothrombin time. *Am. J. Clin. Path.* 17: 405, 1947.
- <sup>7</sup> ASTUP, T. The biochemistry of blood coagulation. *Acta physiol. Scand.* 7: Suppl. 21, 1944.
- <sup>8</sup> GLAZKO, A. J. AND FERGUSON, J. H. Kinetics of thrombin inactivation as influenced by physical conditions: trypsin and serum. *J. Gen. Physiol.* 24: 169, 1940.
- <sup>9</sup> OWREN, P. A. The coagulation of blood: investigation on a new clotting factor. *Acta Med. Scand.* Suppl. 194, 1947.
- <sup>10</sup> FERGUSON, J. H. AND GLAZKO, A. J. Heparin and natural antithrombin in relation to activation and assay of prothrombin. *Am. J. Physiol.* 143: 47, 1941.
- <sup>11</sup> CONLEY, C. L. AND MORSE, W. I. Thromboplastic factors in the estimation of prothrombin concentration. *Am. J. M. Sc.* 215: 158, 1948.
- <sup>12</sup> TOCANTINS, L. M. Relation of contacting surface and an anticephalin activity to maintenance of fluidity and coagulability of blood. *Blood* 1: 156, 1946.
- <sup>13</sup> STERNBERGER, L. A. The stabilization of thrombin in plasma: development of a simple two-stage method for the determination of prothrombin. *Brit. J. Exper. Path.* 28: 168, 1947.
- <sup>14</sup> ———. Hemostasis with an easily prepared stable thrombin solution. *J. Clin. Path.* 1: 229, 1948.
- <sup>15</sup> LONG, M., HURN, M. AND BARKER, N. W. Effect of heparin on prothrombin time. *Proc. Staff Meet. Mayo Clinic* 21: 225, 1946.

- <sup>13</sup> OVERMAN, R S, STAHLMAN W A, SULLIVAN W S, HUEBNER C F, CAMPBELL, A C AND LINK H. P. Studies on the hemorrhagic sweet clover disease. VII. The effect of 3,3'-methylene bis (4-hydroxycoumarin) on the prothrombin time of the plasma of various animals. *J Biol Chem* **142** 941 1942.
- <sup>14</sup> WITTS L. J. Disturbances in the coagulation of blood. *Glasgow M J* **137** 57 1942.
- <sup>15</sup> HURN, M AND MANV, F D. The determination of prothrombin (two-stage method) and anti-thrombin using commercially available reagents. *Am J Clin Path* **17** 741 1947.
- <sup>16</sup> WARNER, E D, BRINKHOUS K M, AND SMITH, H P. Quantitative study on blood coagulation. Prothrombin fluctuation under experimental conditions. *Am J Physiol* **114** 667 1936.
- <sup>17</sup> SMITH H P, WARNER E D AND BRINKHOUS K M. Prothrombin deficiency and bleeding tendency in liver injury (chloroform intoxication). *J Exper Med* **66** 801 1937.
- <sup>18</sup> ALLEN, E. V. The clinical use of anticoagulants. Report of treatment with dicumarol in 1686 post-operative cases. *J A M A* **134** 323 1947.
- <sup>19</sup> BUTSCH, W L, AND STEWART J D. Clinical experiences with dicoumarin. *J A M A* **120** 1025, 1942.
- <sup>20</sup> LEVAN, J B. Dicumarol therapy. *Ann Int Med* **25** 941 1946.
- <sup>21</sup> AGGELER P M. Heparin and dicumarol—anticoagulants: their prophylactic and therapeutic uses. *California & West Med* **64** 71 1946.

# THE EFFECT OF FOLIC ACID ON THE TOXICITY OF ITS ANALOGUE 4-AMINOPTEROYLGLUTAMIC ACID (AMINOPTERIN)

By GEORGE M. HIGGINS, PH D

*With the technical assistance of MURIEL STEMBER AND HARRY MONSEN*

EVER since Woods<sup>1</sup> described a competition between sulfanilamide and para aminobenzoic acid for an enzyme system related to the use of this vitamin in metabolic processes, widespread interest has centered on the synthesis of analogues which are chemically related to a metabolite but which seriously interfere with its normal function. There are now available a large number of these metabolite antagonists<sup>2</sup> which in very minute amounts seriously interfere with the functions of cell catalysts, such as vitamins and hormones.

Such antagonists or displacing agents have now been synthesized for every known member of the vitamin B-complex. Martin, Tolman and Moss<sup>3</sup> prepared the first antagonist to pteroylglutamic acid. They produced methylfolic acid, which was shown to be an effective displacer of folic acid with a ratio of inhibitor to metabolite of 1:150. The growth-promoting action of pteroylglutamic acid for *Streptococcus faecalis* R was antagonized by this analogue.

Franklin, Stokstad, Belt and Jukes<sup>4</sup> fed a crude antagonist, prepared by Hultquist and Smith, to weanling rats. This preparation accelerated and intensified the signs of pteroylglutamic acid deficiency in their rats, lowering hemoglobin levels and granulocyte counts and seriously impairing the maturation of cells in the bone marrow. These effects were all completely prevented, however, by the addition of suitable amounts of pteroylglutamic acid to the diet. Similar results were obtained by giving this same antagonist to mice and to chicks.<sup>5</sup> Pteroylglutamic acid in appropriate amounts also prevented the appearance of this syndrome in these animals. Welch and colleagues<sup>6</sup> provided the same crude antagonist to a pig which was fed a purified diet. They noted a retarded growth rate, alopecia, anorexia, profuse diarrhea and severe anemia. The interference with normal metabolism by the antagonist was removed by giving a crude source of extrinsic factor, essentially free of pteroylglutamic acid, together with normal human gastric juice.

More recently, another analogue of the vitamin, 4-aminopteroylglutamic acid, known as aminopterin, has been synthesized<sup>7</sup> and experimentally tested on mice.<sup>8</sup> This analogue was produced by the replacement of the hydroxy group of the pteridine ring by an amino group, resulting in a much more potent analogue than the 7-methylfolic acid produced by Martin and colleagues.

Since conjugates of folic acid—namely pteroyltriglutamic acid and pteroyldiglutamic acid—have been shown to produce an acceleration of the leukemic process in children, it was concluded that the use of antagonists of pteroylglutamic acid in such cases was certainly indicated. Accordingly, Farber and col-

leagues<sup>9</sup> reported their early results of the use of the more recently synthesized antagonist, 4-aminopteroylglutamic acid, in the treatment of a series of 16 children who had acute leukemia. Marked clinical improvements were noted, and influences were exerted on the immature cells of the peripheral blood, the spleen and lymph nodes. However, the toxic effects which accompanied the use of analogue were severe, including stomatitis and early ulceration.

Stickney, Hagedorn, Mills and Cooper<sup>10</sup> reported that administration of this analogue produced remissions in the clinical course of certain patients who had acute leukemia. In some cases, however, improvement was not elicited. Toxic symptoms, including stomatitis, diarrhea, alopecia and deafness, were recorded. Jacobson, Levin and Holt<sup>11</sup> studied 10 patients with acute leukemia who received aminopterin or methopterin (10-methylpteroylglutamic acid). They concluded that methopterin was superior to aminopterin in the treatment of such leukemias in view of the fact that it was less toxic.

Pierce and Alt<sup>12</sup> reported their results with aminopterin in a series of cases of acute leukemia. Remissions characterized by a severe marrow aplasia followed by rapid regeneration were obtained in 5 of the 11 cases. Berman, Axelrod, Vonder-Heide and Sharp<sup>13</sup> reported their results with the use of aminopterin in 9 patients with chronic leukemia. Although they recognized definite hematologic effects, subjective improvement in any patient was not claimed. They, too, described the toxic effects which followed the administration of the drug.

Since all reports to date indicate that the severe toxic reactions which ensue on administration of this analogue must restrict any extended clinical use of it in spite of its therapeutic value, we undertook a study, in white rats, of some of the toxic manifestations of the analogue together with the modifications of those reactions which were induced when folic acid was given together with aminopterin.

## METHODS

Fifty six young healthy male white rats weighing from 110 to 130 Gm. were selected from our Institute colony. These were arranged into seven groups of 8 animals each so that the average weight of rats composing each group was essentially alike. All animals were maintained in metal cages on open meshwork screens thus greatly restricting coprophagy. Our standard laboratory ration was provided ad libitum and water was available at all times in water bottles attached to the cages.

Six of the seven groups served as test groups and one as an uninjected control. Aminopterin was given intraperitoneally in amounts equivalent to 50 micrograms daily and folic acid was given by stomach tube daily. The various test groups were arranged as follows: each animal in group A received aminopterin alone. Each animal in group B received the same amount of aminopterin plus 5 mg. of folic acid. Group C received aminopterin plus 10 mg. of folic acid. Group D received aminopterin plus 20 mg. of folic acid. Group E received aminopterin plus 30 mg. of folic acid. Group F received aminopterin plus 50 mg. of folic acid. The animals of Group G were given neither aminopterin nor folic acid and served as a normal control group.

At the end of the sixth day the heart blood of all surviving animals was sampled and the white blood and leukocyte tabulations and the differential distributions were recorded. Each animal was kept by etherization and the spleen, adrenals and thymus were removed and weighed on a precision balance.

<sup>4</sup> 4-aminopteroylglutamic acid was made available for our use by the Lederle Laboratories, New York, to whom we are extremely grateful.

Bone marrow imprints were made of samples of marrow obtained from the distal third of the femur. These imprints were stained by the May-Grünwald-Giemsa technic.

## RESULTS

1 *Body Weight Changes* The changes in the weights of these test animals given aminopterin and the influences exerted by varying daily amounts of folic acid are shown in figure 1. For the first three days, increases of weight were recorded for all animals, but those receiving the analogue without the vitamin gained only 2 Gm. during that three-day period. Greatest gains were recorded by the animals receiving the analogue plus 5 mg. of folic acid, although all vitamin-supplemented rats gained more than the controls, which were fed the standard ration.

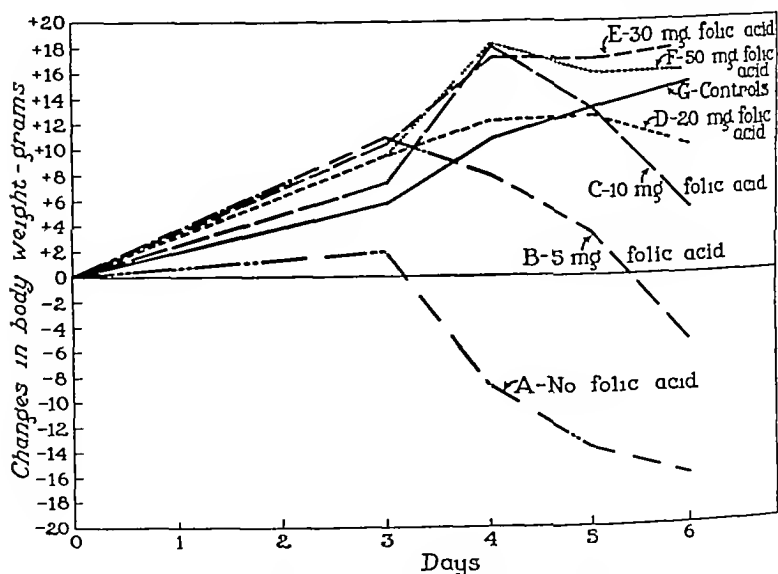


FIG. 1.—Changes in body weights of animals receiving aminopterin and those receiving aminopterin plus varying amounts of folic acid.

and given neither analogue nor vitamin. The data indicate, for the first three days, that the analogue was not seriously toxic and that even the smallest amounts of the vitamin counteracted its effects.

After the third day, however, the toxicity of the analogue was clearly indicated. A severe loss of weight occurred on the fourth day in all animals receiving the analogue alone (group A), and 5 mg. of folic acid was ineffective in preventing loss of weight. Gains of weight, however, were recorded for all animals receiving the larger amounts of vitamin. Ten milligrams of folic acid daily failed to inhibit the toxic effects of the analogue after the fourth day, and 20 mg. of the vitamin did not prevent the loss of weight induced after the fifth day. Thirty milligrams and 50 mg. of the vitamin when given with the analogue maintained

body weights during the fifth and six days, but increases of weight were not recorded

2 *Gross Appearance* The marked contrast in the appearance of animals receiving aminopterin alone and those receiving the analogue plus the vitamin is clearly portrayed in figure 2a and b. The rough coat, the stained hair, the encrusted eyelids and ears, and the extreme diarrhea were all prevented, during the six-day test period, by the administration of 30 mg of folic acid daily by mouth together with the daily intraperitoneal administration of aminopterin.



FIG. 2a—Animal receiving aminopterin without folic acid. b Animal receiving aminopterin plus 30 mg of folic acid daily for six days.

3 *Food Intake* Extreme anorexia was not evident until the third day of the test period, but the average intake of all animals taking the analogue without the vitamin was less than 1 Gm a day (fig 3). The addition of 5 mg of the vitamin stimulated the appetite only slightly, although 10 mg daily increased the food intake to more than three times that of animals taking the analogue alone and 30 mg of folic acid, when given with the analogue, so stimulated the appetite as to maintain an average food intake in excess of 9.0 Gm daily. However in the amounts given, folic acid did not so nullify the effects of its analogue as to maintain appetites in any test animal equal to those of animals constituting the untreated control group.

4 *The Weight of the Adrenal Glands* The adrenal glands invariably reflect onto-

ward reactions of an organism to toxic substances. Hyperplasia of the adrenal cortex, together with marked atrophy of the thymus, constitutes part of a syndrome embracing the reactions of an organism to unfavorable environments induced by a number of different factors. In this test, too, of the toxicity of aminopterin, the increased weights of the adrenal glands indicated an untoward reaction of the animals toward the drug.

The combined weights of the adrenal glands recorded at necropsy of all surviving animals of all seven groups on the sixth day are graphically portrayed (fig. 4). The average combined adrenal gland weight of all control animals was 20.0 mg, but in the group given aminopterin alone (A) the average combined

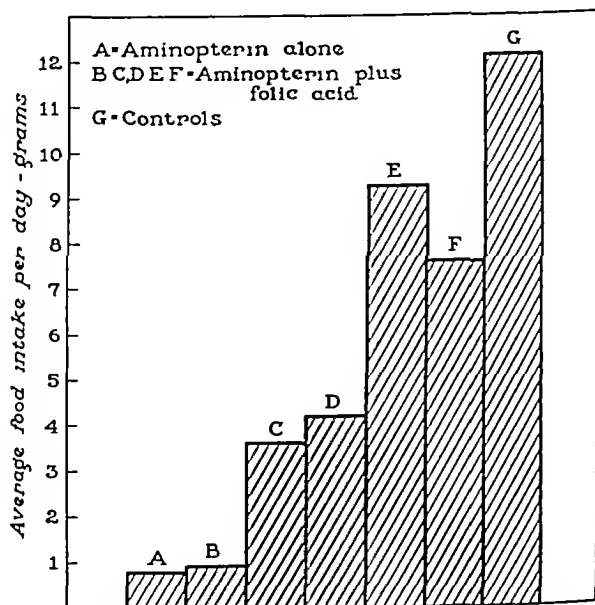


FIG. 3—The degree of anorexia induced in animals by giving aminopterin and by giving aminopterin plus varying amounts of folic acid.

weight of the adrenals was slightly in excess of 51.0 mg. The addition of only 5.0 mg. of folic acid daily reduced the average combined weight of the adrenal glands to 41.5 mg., and the administration of 30 mg. of folic acid daily to animals given the analogue resulted in restricting the weight of both adrenal glands to 28.0 mg. The administration of 50 mg. of the vitamin daily was less effective than that of 30 mg. in restricting hyperplasia.

5. *The Weight of the Thymus* Atrophy of the thymus constitutes a part of the syndrome which ensues within an animal on the administration of toxic or harmful substances, so that atrophy of the thymus usually accompanies adrenal hypertrophy. The data obtained by weighing the thymus of all animals at necropsy are

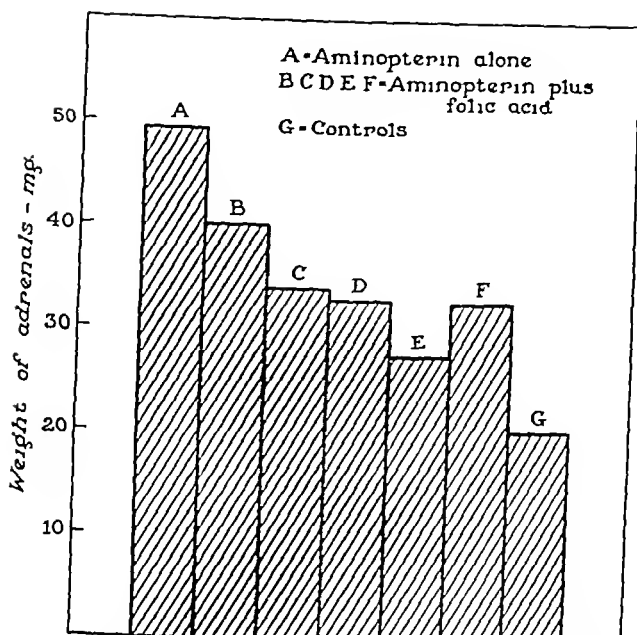


FIG 4—Weights of the adrenal glands of animals receiving aminopterin and those receiving aminopterin plus varying amounts of folic acid, when the animals were killed on the sixth day

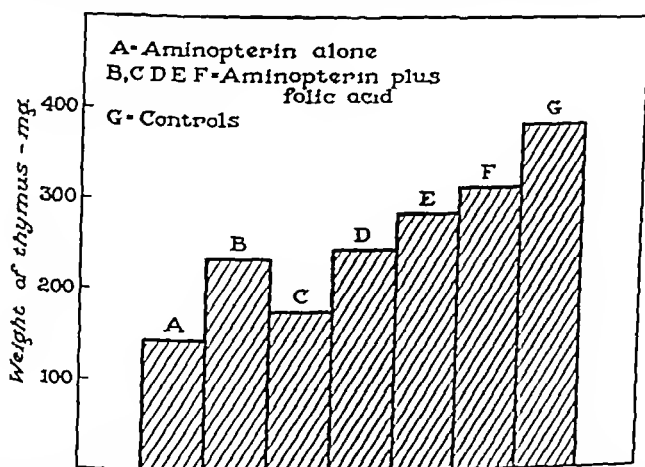


FIG 5—Weights of the thymus of animals receiving aminopterin and those receiving aminopterin plus varying amounts of folic acid when the animals were killed on the sixth day

recorded in figure 5, and photographs of the glands of 2 animals receiving aminopterin and of 2 animals receiving aminopterin plus 30 mg of folic acid daily are shown (fig 6)



The average weight of the thymus of the 8 control animals (group G) was slightly less than 400 mg, while that of animals receiving aminopterin alone (group A) was slightly less than 150 mg. The range of thymus weight of animals within group A extended from 59.6 mg to 296.8 mg. The administration of the various amounts of the vitamin (pteroylglutamic acid) had a considerable influence on restricting the extent of the atrophy, but the daily administration of 50 mg of the vitamin resulted in maintaining an average thymus weight of 320.5 mg, a figure considerably less than that recorded for the control animals (group G).

6 *The Weight of the Spleen* The size of the spleen is ordinarily not a reliable criterion for recording systemic reactions. A vascular organ, with capillaries and venous sinuses, the spleen is subject to rather rapid changes in size, correlated with the extent to which fluid or other blood constituents are sequestered within it. Splenic size varies considerably with the anesthetic agent used. Ether has a constricting effect on the organ, while pentobarbital sodium (nembutal) will ordinarily dilate the sinuses and enlarge it.



FIG. 6—Thymus of animals receiving aminopterin without folic acid (left) and of animals receiving aminopterin plus 30 mg of folic acid daily (right)

Since the data herein reported were obtained on animals correspondingly etherized, there is reason to believe that they constitute a reasonably accurate response of the spleen to the experimental restrictions imposed by the study. The data assembled on the weights of the spleens of all animals are given in figure 7.

Since aminopterin inhibits blood cell formation, it is obvious that the spleen, a blood-forming organ, would be affected by this drug. Our data indicate wide variability in the size of the spleen in animals receiving aminopterin alone (group A). In some instances the spleen appeared as a narrow pale band of tissue, weighing as little as 91.8 mg, in others it was more nearly normal, and in one animal it weighed 408.4 mg. The average weight of the spleen encountered for group A was 266.0 mg, which is considerably less than the average weight recorded for the control group G, namely, 600.0 mg. Photographs of the spleens of 2 animals receiving the drug and of those of 2 animals receiving the drug plus the vitamin are shown (fig. 8).

As in the data assembled on adrenals and thymus, so in those recorded for the weights of the spleens of the various groups, folic acid in the amounts given

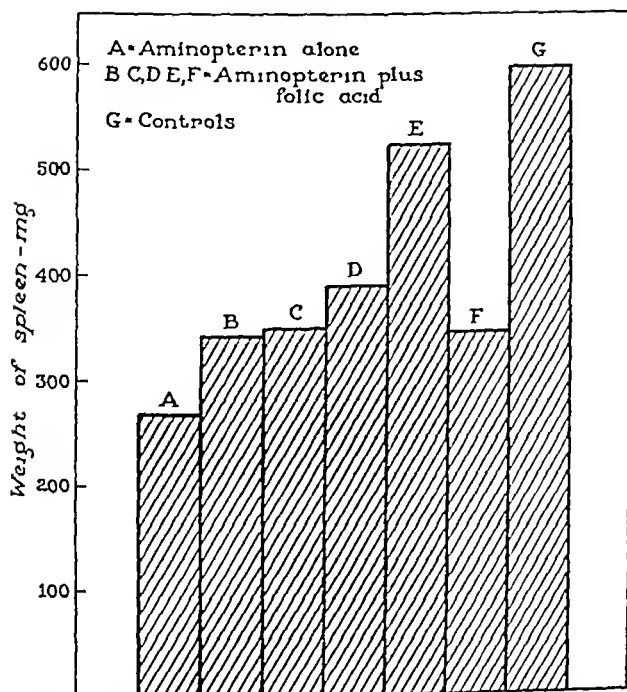


FIG 7—Weights of the spleens of animals receiving aminopterin and those receiving aminopterin plus varying amounts of folic acid when the animals were killed on the sixth day



FIG 8—Spleens of animals receiving aminopterin without folic acid (left) and of animals receiving aminopterin plus 30 mg of folic acid daily (right)

The average weight of the thymus of the 8 control animals (group G) was slightly less than 400 mg, while that of animals receiving aminopterin alone (group A) was slightly less than 150 mg. The range of thymus weight of animals within group A extended from 59.6 mg to 296.8 mg. The administration of the various amounts of the vitamin (pteroylglutamic acid) had a considerable influence on restricting the extent of the atrophy, but the daily administration of 50 mg of the vitamin resulted in maintaining an average thymus weight of 320.5 mg, a figure considerably less than that recorded for the control animals (group G).

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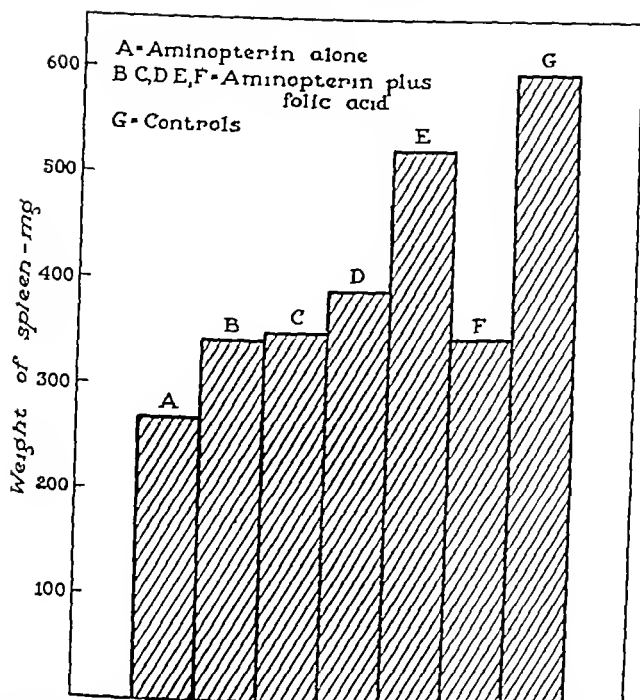


FIG 7—Weights of the spleens of animals receiving aminopterin and those receiving aminopterin plus varying amounts of folic acid, when the animals were killed on the sixth day

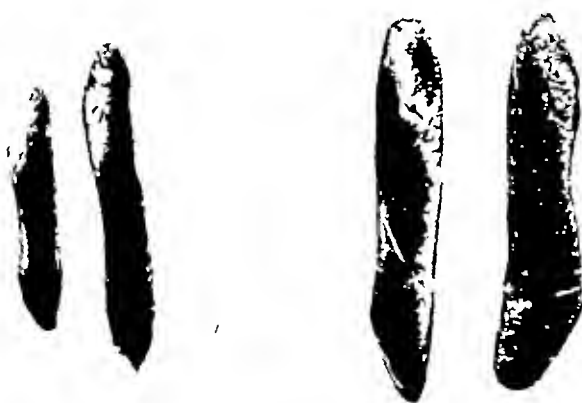


FIG 8—Spleens of animals receiving aminopterin without folic acid (left) and of animals receiving aminopterin plus 30 mg of folic acid daily (right)

maintained more nearly normal weights than in group A. Five milligrams of the vitamin exerted a considerable effect, but 30 mg daily maintained in group E an average spleen weight (525.0 mg) closely approaching the average weight in the untreated controls.

7 *The Changes in Leukocytes in the Peripheral Blood* Aminopterin markedly restricted the total number of leukocytes in the peripheral blood of these young rats. In a series of 8 rats, selected from the same age group as those used to test the effects of this analogue, and known as a preinjection control group, the total leukocyte count was 14,000 cells per cubic millimeter of blood. Of these, approxi-

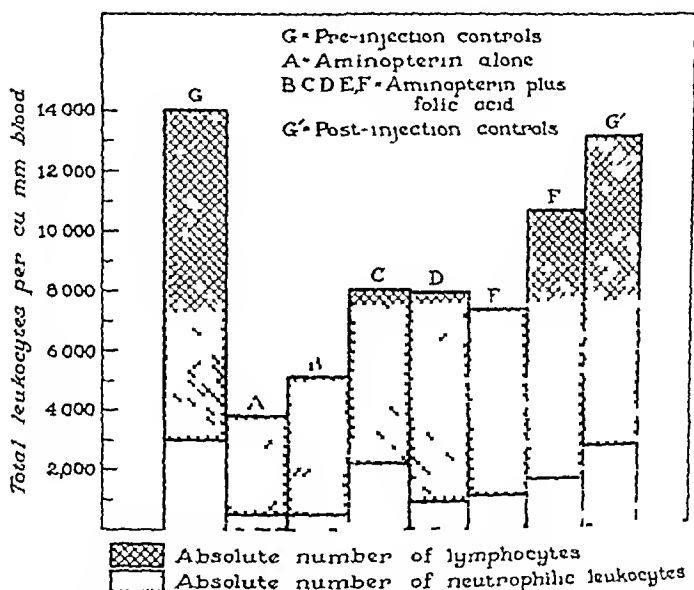


FIG. 9.—Distribution of leukocytes in the peripheral blood of normal animals, those receiving aminopterin, and those receiving aminopterin plus varying amounts of folic acid daily, on the sixth day of the experiment.

mately 11,000 were lymphocytes and 3,000 were neutrophilic leukocytes, but small percentages of eosinophilic and basophilic leukocytes and monocytes were present. The data herein reported are restricted to a consideration of the reduction in the absolute numbers of lymphocytes and neutrophilic leukocytes per cubic millimeter of blood imposed by aminopterin and of the influences exerted by giving the varying amounts of folic acid to such aminopterin-treated animals (fig. 9).

Six days of the intraperitoneal injection of the analogue, in the amounts selected, reduced the total numbers of lymphocytes and neutrophilic leukocytes to 3,800 per cubic millimeter of blood. Accepting the data of the preinjection control group as standard, or representative of the blood of the test groups before injec-

tions began, it is obvious that aminopterin restricted both categories of white blood cells. The total number of lymphocytes dropped from a level of 11,000 to one of 3,300 per cubic millimeter, and the total number of neutrophilic leuko-

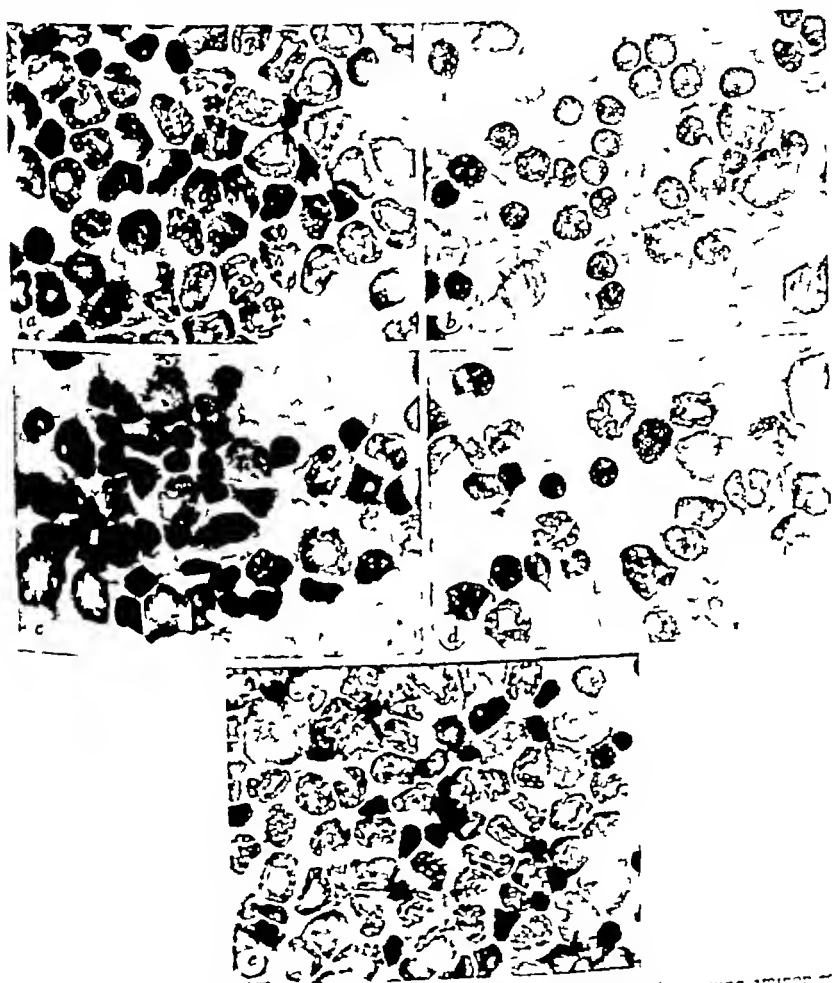


FIG. 10.—Imprints of the femoral bone marrow. *a* Normal rat. *b* Animal receiving aminopterin daily for six days. *c* Animal receiving aminopterin daily for six days plus 5 mg. of folic acid daily. *d* Animal receiving aminopterin daily for six days plus 30 mg. of folic acid daily. *e* Animal receiving aminopterin daily for six days plus 50 mg. of folic acid daily.

cytes dropped from a level of 3,000 to one of 500 cells per cubic millimeter. The smallest amount of the vitamin administered (5 mg. daily) had no effect whatever on the total number of neutrophilic leukocytes but did considerably elevate

the total lymphocyte count. For reasons difficult to interpret, 10 mg of the vitamin daily resulted in a considerable increase in the total numbers of neutrophilic leukocytes—up to 2,200 cells per cubic millimeter—and 5,800 lymphocytes for the same amount of blood, while those animals receiving the larger amounts of the vitamin—20, 30, and 50 mg respectively—had lesser numbers of neutrophilic cells. Increased numbers of lymphocytes, however, were found to occur in animals receiving 50 milligrams of the vitamin daily (group F), nearly approximating the lymphocyte level which obtained in the postinjection control group (G).

8 *The Bone Marrow* Imprints of bone marrow from the distal end of the femur were obtained at necropsy from representative animals of each group. These were stained by the May-Grunwald-Giemsa technic. Figure 10 rather well portrays the changes which ensued when the analogue was given and the modifications of these changes which followed the administration of the varying amounts of the vitamin together with the analogue.

Normal rat marrow contains cells of both the erythroid and the myeloid series in varying stages of maturation (fig. 10a). Attempts were not made in the present study to establish changes in the percentage distributions of the various cellular categories, but it was clearly obvious that marked inhibitory changes were in cited. A glance at the marrow preparations of animals receiving the analogue for the six days (fig. 10b) shows how completely the maturation of the myeloid cells had been suspended. These imprints show that the marrow, in the region examined, was composed of cells almost exclusively erythroid, although a very few early myeloid forms were present.

The administration of 5 mg of folic acid to animals receiving the analogue in cited a slight myeloid response (fig. 10c), although the smears indicated that the marrow was still largely erythroid. But there were larger numbers of myelocytes and metamyelocytes in the imprints stimulated by the added small amount of vitamin. The response of the bone marrow to the increasing amounts of folic acid was more nearly proportional to the amounts injected than in any other organ observed. As the amounts of the vitamin were increased, the percentages of myeloid cells in the marrow were correspondingly elevated. When 30 mg of the vitamin were given, larger numbers of immature myeloid cells were identified in the imprints, and their maturation resulted in many fully mature granulocytes (fig. 10d). The oral administration of 50 mg of the vitamin resulted in the retention of a marrow pattern which was entirely normal (fig. 10e). The numbers of mature granulocytes appeared even to exceed those accepted as a normal distribution.

#### COMMENT

This study, incomplete though it is with respect to all the many other toxic reactions induced by the analogue, was undertaken to determine the extent to which the vitamin, folic acid, would counteract the untoward effects of the antagonist, 4-aminofolic acid. The therapeutic effects of this folic acid antagonist are of sufficient value clinically to warrant studies directed toward a modification of the toxic symptoms which accompany its use. If the analogue could be so modified as to

restrict the extreme degrees of enteritis which develop on its administration and yet retain the marked inhibitory effect on the development of myeloid cells in the bone marrow, its effective clinical use would be assured.

By the administration of the vitamin together with the analogue we have demonstrated satisfactorily that, for a certain period, a given amount of vitamin will completely nullify a given amount of antagonist. The toxic reactions which were so severe in animals of group A, given aminopterin alone, included anorexia, atony of the entire gastro-intestinal tract with gastric and intestinal distention, marked diarrhea, adrenal hyperplasia and atrophy of the thymus, spleen and bone marrow, with resulting leukopenia.

Partial remission of these disorders was obtained by giving small amounts of the vitamin daily, but larger amounts, 30 mg. daily, essentially inhibited the destructive effectiveness of the analogue in all of the categories enumerated. To be sure, a complete return to normal gland weights and to normal blood levels was not attained in all animals receiving that amount of folic acid daily, yet the gross appearance of the animals, the character of the gastro-intestinal tract, and the restored appetite all certified to the general deduction that, for the six-day test period, it required 30,000 micrograms of folic acid daily to counteract the toxic effects of 50 micrograms of 4-aminofolic acid daily. This is a ratio of inhibitor to metabolite of 1:600.

The toxic effects of aminopterin are not immediately evident on its administration. For three days, animals showed no ill effects of its intraperitoneal administration. Then, extremely rapidly, even over night, all the foregoing toxic manifestations may present themselves. This delay in the onset of symptoms, we presume, is due to the presence of a reserve of folic acid in the organism at the outset of the experiment. It may be that as soon as the analogue had destroyed this reserve of the vitamin, the toxic symptoms appeared. And yet these symptoms cannot all be ascribed to a folic acid deficiency, for we are not aware that they ensue to this extent in animals fed diets deficient in folic acid. Nevertheless, they did not develop when large amounts of the vitamin were fed, for the six-day period, together with aminopterin.

Although this report covers a short-time study of the relationship of the vitamin, folic acid, to the toxicity exerted in rats by the antagonist, aminopterin, and shows unquestionably the inhibition exerted by the vitamin for a six-day period, yet we have other data which show that this inhibition was not effective indefinitely. Our results show that the characteristic syndrome incited by the amounts of aminopterin we administered, was not inhibited for periods longer than fourteen days by giving 30 mg. of folic acid daily. We have not extended our observations to include the results of giving 50 mg. of the vitamin for the longer period. Reasons for the failure of the vitamin to inhibit the antagonist for longer periods are not clear. It may be that further increase of the amounts of folic acid could well antagonize the analogue, so as to restrict permanently the onset of the toxic effects. There is need, therefore, for much more research on the functional interrelationships of this vitamin and its powerful antagonist, aminopterin.



## SUMMARY AND CONCLUSIONS

A study of some of the toxic reactions of 4-aminofolic acid together with the modifications of these reactions induced by giving varying amounts of folic acid daily to white rats is reported

Seven groups of young male rats ranging in weight from 110 to 130 Gm were arranged. Aminopterin (4-aminofolic acid) was given intraperitoneally, in amounts equivalent to 50 micrograms daily, to all rats of six of these seven groups. Folic acid was given by mouth to these animals in such amounts as to provide 5, 10, 20, 30 or 50 mg daily to each animal respectively of five of the six groups receiving aminopterin. One group received the analogue without the vitamin. One group of 8 animals received neither the vitamin nor its analogue.

Observations continued for six days, when all surviving animals were killed and necropsy was performed. Data were assembled on the appetite, body weights, the weights of adrenals, thymus and spleen, the distribution of leukocytes in the peripheral blood and the changes in the bone marrow. The following conclusions seem warranted:

1. Aminopterin is extremely toxic and incites within six days anorexia, extreme diarrhea with atony of the entire gastro-intestinal tract, adrenal hyperplasia, atrophy of the thymus and spleen, and an inhibition to development of myeloid cells in the bone marrow.

2. Small amounts of folic acid are essentially without effect on the toxicity of that amount of the analogue we chose to administer.

3. Larger amounts of folic acid daily (30 mg) proved effective in essentially inhibiting the development of the severe toxic reactions for the six-day period.

4. The severe toxicity of aminopterin does not manifest itself until the third or fourth day of its daily administration. The onset of these symptoms is thereafter extremely rapid.

5. Thirty milligrams of folic acid daily will not indefinitely counteract the toxic symptoms induced by 50 micrograms of aminopterin. In our experience, within twelve to fourteen days, the characteristic syndrome will appear in spite of the continuous administration of the vitamin.

## REFERENCES

1. WOODS D. D. The relation of p-aminobenzoic acid to the mechanism of the action of sulphanilamide. *Brit J Exper Path* 21 74 1940
2. ROBLIN R. O. JR. Metabolic antagonists. *Chem Rev* 38 255 1946
3. MARTIN, G. J. TOLMAN LEO AND MOSE JACK. d(-)methylfolic acid displacing agent for folic acid. *Arch Biochem* 12 318 1947
4. FRANKLIN A. L. STOKSTAD E. L. R. BELT, MAROARET AND JUKES T. H. Biochemical experiments with a synthetic preparation having an action antagonistic to that of pteroylglutamic acid. *J Biol Chem* 169 427 1947
5. ——— AND JUKES T. H. Acceleration of pteroylglutamic acid deficiency in mice and chicks by a chemical antagonist. *Proc. Soc. Exper Biol & Med* 65 368 1947
6. WELCH A. D. HEINLE R. W. SHARPE GEORGE GEORGE, W. L. AND EPSTEIN MARTIN. Chemical antagonism of pteroylglutamic acid in a pig. Hematopoietic effect of extrinsic and intrinsic factors. *Proc. Soc. Exper Biol & Med* 65 364 1947

- <sup>7</sup>SEGER D R SMITH J M, JR AND HULTQUIST M E Antagonists for pteroylglutamic acid J Am Chem Soc 69 2567 1947
- <sup>8</sup>FRANKLIN A L, STOKSTAD E L R AND JUKES T H Observations on the effect of 4 amino-pteroyl glutamic acid on mice Proc Soc Exper Biol & Med 67 398 1948
- <sup>9</sup>FARBER SIDNEY DIAMOND, L. K. MERCER, R D SYLVESTER R F AND WOLFF J A Temporary remissions in acute leukemia in children produced by folic acid antagonists: 4 amino-pteroyl glutamic acid (aminopterin) New England J Med 238 787 1948
- <sup>10</sup>STICKNEY J M HAGEDORN A B, MILLS S D AND COOPER TALBERT Changes in blood and bone marrow in acute leucemia induced by aminopterin J Lab & Clin Med 33 1481 1948
- <sup>11</sup>JACOBSON, W LEVIN W C AND HOLT GORDON Observations on the treatment of acute leucemias with analogues of folic acid J Lab & Clin Med 33 1641 1948
- <sup>12</sup>PIERCE MILA AND ALT HOWARD Treatment of acute leucemia with aminopterin (4 amino-pteroyl glutamic acid) J Lab & Clin Med 33 1642, 1948
- <sup>13</sup>BERMAN LAWRENCE AXELROD A R, VONDERHEIDE E C AND SHARP E A Treatment of chronic leucemia with a folic acid antagonist J Lab & Clin Med 33 1643 1948

# ERYTHROCYTOPOIESIS IN THE NERVOUS SYSTEM OF THE EMBRYONIC RAT

By MATTHEW BLOCK, M D , Ph D

IN THE course of experiments to influence embryonic hematopoiesis in rats<sup>1</sup> the constant occurrence of erythrocytopoiesis in various parts of the embryonic central and peripheral nervous system was observed. The following is a description of erythrocytopoiesis occurring in this site and indicating its origin and relation to erythrocytopoiesis in other sites of the rat embryo and a discussion of the implications of the existence of this previously undescribed site of erythroblastic activity. The histologic description applies to both normal embryos and to those subjected to various stimuli to modify hematopoiesis since the microscopic appearance of the tissues in these two groups was identical in all embryos of corresponding age.

## MATERIAL AND METHODS

The ages of the embryos and the stimuli used to modify hematopoiesis have been described in detail in a previous investigation.<sup>1</sup> In brief, there were 25 rat embryos in the experimental series varying from 8 to 18 days of development with most of the embryos being 14 to seventeen days of development. The pregnant rats were subjected to the following stimuli in the experimental series, injections of saprotoxin, phenylhydrazine, dinitrophenol, concentrated liver extract and Evans blue, feedings of thyroid extract, exposure to chloroform vapor, and repeated withdrawal of blood by cardiac puncture.

At least one normal embryo was studied for each day of intrauterine development until birth on the twenty-first day of gestation. During the fourteen to seventeen day period at least two normal embryos were studied for each day of development. In addition, in the laboratory of Professor William Taliaferro of the Parasitology Department, rats known to be Bartonella-free were mated and 16 and 17 day embryos were obtained for study.

The embryos were fixed for two to six hours in Zenker-formol, embedded in nitro-cellulose and cut serially at 8 micra. The slides were stained with hematoxylin eosin-azure II and Mallory-azan. Some slides were impregnated by means of the Bielschowsky technic for reticular fibers and counterstained with Mallory azan.

## MICROSCOPIC OBSERVATIONS

*Eleventh Day* The central nervous system consists of a tube whose walls are made up of undifferentiated neuro-epithelium, and which is separated from the

From the Department of Medicine University of Chicago Chicago Ill

This work was aided by a grant from the Dr. Wallace C. and Clara A. Abbot Memorial Fund of the University of Chicago. Supported in part by a grant from the American Cancer Society on recommendation of the Committee on Growth of the National Research Council.

surrounding mesenchyme by an external limiting membrane. The cerebrospinal and sympathetic ganglia are also made up of an undifferentiated neuro-epithelium. Vascularization of the central and peripheral nervous system has not yet begun.

The cells of the neuro-epithelium and of the mesenchyme resemble each other quite closely but may be distinguished with some difficulty. The former have a smooth homogeneously stained cell body which has a rectangular or oblong shape, the latter have a finely vacuolated cell body that is irregularly stellate in shape. The chromatin particles in the nuclei of the neuro-epithelial cells are heavier and fewer in number than in the mesenchymal cells.

*Twelfth Day.* Neuroblasts, are now recognizable because of the presence of the characteristic oval nuclei with smooth fine nuclear membranes and round nucleoli. In addition, in favorably oriented sections naked axones may be traced from the cell bodies of these cells into the mesenchyme. The supporting cells (spongioblasts) of the nervous system at this stage have not differentiated into the various types of glia. In contrast to the mesenchymal cells, the neuro-epithelial cells are arranged in epithelial sheets, even in the ganglia. The neuro-epithelial cells may still be discriminated from the mesenchymal cells by the previously described criteria except at the edge of the ganglia and along the origin of the nerve roots where the neuro-epithelial cells lose their sheetlike arrangement and the individual cells become somewhat stellate.

By the twelfth day capillaries have begun to grow into the central nervous system and peripheral ganglia. Numerous mitoses are present in the endothelial cells. At this point the external limiting membrane of the central nervous system seems to be pushed in by the capillary endothelium and reflected over the outer surface of the endothelium. In the central nervous system, the endothelial cells rest directly against the surrounding neuro-epithelium. At this time there are no perivascular spaces and no perivascular mesenchymal cells (fig. 1a and b). There is no external limiting membrane in the ganglia and the vessels seem to penetrate directly in between the neuro-epithelial cells.

The endothelial cells (fig. 1a) at this stage of development may be separated from the supporting neuro-epithelial cells (fig. 1b) by the following criteria. The nucleus of the endothelial cell (fig. 1a) has finer, sharper chromatin particles and a more irregular and larger nucleolus than the neuro-epithelial cells (fig. 1b). The nuclear sap is paler in the endothelial cell nucleus and the nuclear membrane is finer and sharper. The endothelial cell cytoplasm extends parallel to the long axis of the vessel in contrast to the irregularly stellate, poorly demarcated spongioblast cytoplasm. However, it must be emphasized that these differences, although constantly present, are minute, especially in the ganglia. During the remainder of embryonic life the neuro-epithelial cells, except for the Schwann cells and the satellite cells supporting the neurones of the ganglia, may be separated from the mesenchymal cells without difficulty.

At this stage of development, the circulating blood cells are all derived from the blood islands of the yolk sac. Practically all of these cells are basophilic and polychromatophil primitive erythroblasts (fig. 1c) which were formed from the

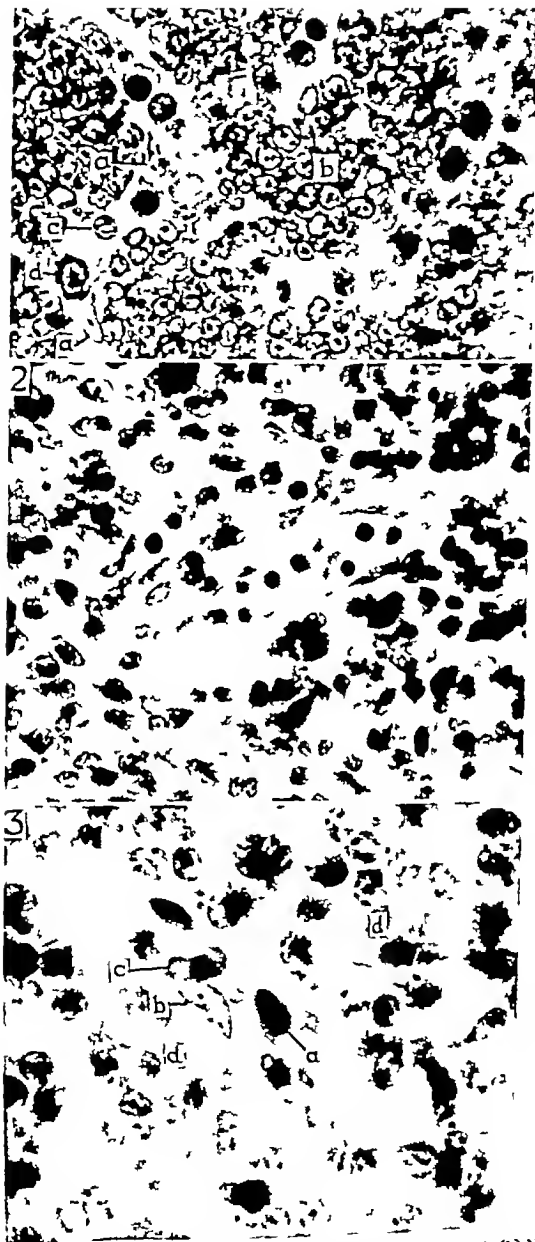


FIG 1 — BRAIN OF A 12 DAY EMBRYO Photograph  $450\times$  (a) Endothelial cell (b) Neuro-epithelial cells (c) Polychromatophil primitive erythroblast (d) Hemocytoblast (e) Polychromatophil primitive erythroblast in mitosis FIG 2 — BRAIN OF A 13 DAY EMBRYO Photograph  $450\times$  FIG 3 — BRAIN OF A 14 DAY EMBRYO Photograph  $450\times$  (a) Perivascular mesenchymal cell (b) Endothelial cell (c) Orthochromatic primitive erythroblast of the circulating blood (d) Neuro-epithelial cells

mesenchyme in the yolk sac after passing through a hemocytoblast (myeloblast) stage<sup>1 2 3</sup> In this process almost all hemocyto blasts have developed into primitive erythroblasts so that only rarely does one find a hemocyto blast (fig 1d) in the circulating blood The erythroblasts continue to increase by mitotic proliferation primarily in the yolk sac vessels but also to a limited extent in the circulating blood (fig 1c)

The body mesenchyme at this time consists exclusively of loosely arranged outstretched mesenchymal cells, there is no evidence of formation of hemocyto blasts or histioid wandering cells (embryonic macrophages), nor is there any hematopoiesis The liver anlage is present, but as yet, manifests no hematopoietic activity The sole hematopoietic organ is the yolk sac, which is at this time the source of all the circulating blood cells of the embryo

*Thirteenth Day* Vascularization of the central and peripheral nervous system has progressed to such an extent that they are supplied with a rich net of anastomosing capillaries The cytologic differences between the endothelial cells and the spongioblasts (supporting cells) of the central nervous system have become more obvious But in the ganglia it is still impossible to distinguish many of the cells of presumed mesenchymal origin from many of the supporting cells of presumed neuro-epithelial origin The Schwann cells ensheathing the nerve roots are still indistinguishable from the surrounding mesenchymal cells

A significant change is observed in the neuro-epithelium of the central nervous system the cells have become much looser in arrangement especially around the vessels (fig 2) This is probably not artefact since it is constantly present in this location and is absent around blood vessels in other parts of the embryo including the ganglia It is the area in which Held's space will be located<sup>4 5</sup> Silver impregnation and Mallory-azan stains fail to demonstrate any reticular or collagenous fibers supporting the vessels at this stage of development

A new feature makes its appearance during the thirteenth or fourteenth day when perivascular mesenchymal cells (fig 3a) are observed along the vessels in the central nervous system As in all other processes, this ingrowth is always more advanced in the anterior than posterior part of the embryo The perivascular mesenchymal cells lie in close contact with the endothelium (fig 3b) between it and Held's space and so occupy the future Virchow-Robin space These cells are first seen along the entrance of the blood vessels into the central nervous system Occasionally a perivascular mesenchymal cell may be seen migrating into the central nervous system as evidenced by the fact that a part of the cell may be found in the central nervous system and the remainder in the surrounding mesenchyme There is no evidence that these cells are derived from the endothelial cells or the spongioblasts, or any of the cells of the circulating blood At this stage Mallory-azan stain and silver impregnation still fail to demonstrate the presence of reticular fibers around the vessels in the central nervous system

The perivascular mesenchymal cells (fig 3b) have a moderately basophilic somewhat vacuolated cytoplasm The nuclei have a heavily stained nuclear membrane and dark compact chromatin particles in a dark nuclear sap They are similar to

the histioid wandering cells (embryonic macrophages) of Maximow which have begun to develop heteroplastically from mesenchymal cells all through the body mesenchyme except that the latter have a more clearly demarcated cell border, a more coarsely vacuolated cytoplasm, and may be irregularly circular in outline instead of outstretched like the perivascular mesenchymal cells

At this stage active proliferation of primitive erythroblasts is still going on in the yolk sac sinuses. Rarely, basophilic definitive erythroblasts and hemocytoblasts may be found extravascularly in the yolk sac.<sup>1</sup> Most of the primitive erythroblasts in the circulation are in the late polychromatophil or orthochromatic stage (fig 3c). Hemocytoblasts (myeloblasts) are extremely rare in the circulating blood. Definite erythrocytopoiesis has begun in the liver. Most of the hematopoietic cells in the liver are hemocytoblasts or very young basophilic definitive erythroblasts. There is no sign of hematopoiesis in the diffuse mesenchyme of the embryo.

*Fourteenth Day* This period of development is marked by an increase in number of the perivascular mesenchymal cells so that almost all of the vessels in the central nervous system display a continuous layer of these cells. Typical histioid wandering cells are also found scattered in small numbers through the neuro-epithelium without any relation to blood vessels in the central nervous system.

The situation in the sympathetic and cerebrospinal ganglia is difficult to elucidate. The capillaries seem to lie directly against the supporting cells of the neuroblasts and only rarely are perivascular mesenchymal cells to be seen except at the edge of the ganglia near the mesenchyme. Here it is still impossible to separate the supporting cells of neuro-epithelial origin from the mesenchymal cells by any of the cytologic techniques used.

*Fifteenth Day* Hemocytoblasts are occasionally found extravascularly for the first time in the central nervous system and ganglia of the embryo. One may find isolated transitional stages between mesenchymal cells and perivascular mesenchymal cells and hemocytoblasts (fig 4a) and between the two former cell types and histioid wandering cells in various parts of the nervous system. The hemocytoblasts and histioid wandering cells are still found primarily in a perivascular location in the central nervous system and near the edge of the ganglia in the peripheral nervous system.

Identical heteroplastic formation of hemocytoblasts and histioid wandering cells is also present throughout the diffuse mesenchyme.

The origin of the erythrocytopoiesis in the central nervous system is easier to trace than in the peripheral nervous system because the erythroblasts are derived from the perivascular mesenchymal cells after passing through a hemocytoblast or histioid wandering cell stage, and because the perivascular mesenchymal cell may be clearly differentiated morphologically from the surrounding spongioblasts. However, in the ganglia the supporting cells of probable neuro-epithelial origin and the mesenchymal cells are so similar as to be hardly distinguishable on cytologic grounds (fig 4b vs c). In general, the mesenchymal cells have sharper, smaller chromatin, paler nuclear sap and larger nucleoli but in many instances these distinctions do not seem to be demonstrable and it is therefore sometimes



FIG. 4.—FIFTH CRANIAL GANGLION OF A 15 DAY EMBRYO. Camera lucida drawing 1800 X. (a) Transitional stage between a mesenchymal cell and a hemocytoblast (b) Neuro-epithelial cell (c) Mesenchymal cell (d) Intravascular orthochromatic primitive erythroblasts

maturation in the nervous system and body mesenchyme at the same level with respect to the anterior-posterior axis in any one embryo.

*Sixteenth to Twenty-first Day.* The precursors of the erythroblasts—the free stem cells (hemocytoblasts and histioid wandering cells)—have begun to transform into definitive erythroblasts (fig. 5c). Apparently all the hemocytoblasts in the nervous system differentiate into erythroblasts, but, as in the loose mesenchyme,<sup>6</sup> some histioid wandering cells remain scattered through the central nervous system.

The maturation of the erythroblasts of the nervous system is entirely similar to that of erythroblasts in the embryonic liver and mesenchyme, and embryonic and adult bone marrow. They are all of the definitive erythroblast series in contrast to the primitive erythroblasts which were formed intravascularly in the yolk sac only<sup>1, 2, 6</sup> and which are the first circulating blood cells of the embryo (figs. 1-5). The more immature basophilic erythroblasts have rather large acidophil nucleoli



and a small amount of chromatin in large vesicular nuclei surrounded by densely basophilic cytoplasm (fig 5c) In the course of maturation the large nucleoli break down into many smaller ones, the chromatin particles begin to assume a checker board pattern and the cytoplasm becomes less basophilic so forming the poly



FIG 5 — LAMINA TERMINALIS OF A 16 DAY EMBRYO Camera lucida drawing 2200 X (a) Neuro-epithelial cells (b) Hemocytoblast (c) Immature definitive basophilic erythroblast (d) Intravascular orthochromatic primitive erythroblast

chromatophil and orthochromatic erythroblasts (figs 5 and 6) Under the low power the nuclei of the latter appear quite dark (fig 7a) Mitoses are quite frequent (fig 6c)

At this stage the erythroblasts may superficially resemble the supporting cells of the nervous system The latter have more delicate chromatin particles, darker



FIG. 6—THORACO-LUMBAR SYMPATHETIC GANGLION OF A 16 DAY EMBRYO. Camera lucida drawing 1800 $\times$ . (a) Neuroblast (b) Polychromatophil definitive erythroblast (c) Polychromatophil definitive erythroblast in mitosis (d) Satellite cell of neuro-epithelial origin

Aggregates of erythroblasts are always found in certain characteristic locations in the nervous system (figs 5, 6 and 7) and sporadically in almost every part of the nervous system. Relatively large foci of erythroblasts are invariably encountered in the lamina terminalis (figs 5 and 7) of the central nervous system.

Smaller foci are found in the thalamus and ventral portions of the cerebral hemispheres. Small foci of erythroblasts are also found all through the rest of the brain and to a very limited extent in the spinal cord. Erythrocytopoiesis is common in the cerebrospinal ganglia, especially anteriorly. Occasional foci are present in the roots of the cerebrospinal and sympathetic ganglia. Large masses of erythroblasts are invariably encountered in the thoraco-lumbar sympathetic ganglia, in some cases even outnumbering the cells of neuro-epithelial origin (fig. 6). Erythrocytopoiesis is rare in the peripheral ganglia in the intestinal tract.

The further development of this erythrocytopoiesis in the nervous system is difficult to trace. During the eighteenth and nineteenth days of development the erythroblasts in the nervous system disappear entirely. At this time collections of degenerating cells are found in the central nervous system corresponding to the



FIG. 7.—LAMINA TERMINALIS OF AN 18 DAY EMBRYO. Photograph 300 X. (2) Focus of erythroblasts.

location of the erythroblasts but, since foci of spontaneous degeneration of the neuro-epithelial and other cells are so common in intrauterine life, it is impossible to determine whether the cells undergoing spontaneous degeneration in the nervous system are solely of neuro-epithelial or of mixed origin.

#### DISCUSSION

The first investigators to stress the hematopoietic potentialities of the mesenchymal cell in localities other than the commonly recognized hematopoietic sites in the embryo were Saxer<sup>3</sup> and Maximow.<sup>4</sup> In 1909 Maximow emphasized the ability of the mesenchymal cell, not only in the areas as yolk sac and liver which are commonly accepted as hematopoietic organs, but all through the diffuse body mesenchyme to develop into free multipotent cells and to give rise to the various blood cells.

Since then, Maximow's concept of the multipotentialities of the mesenchymal cell has been substantiated by numerous reports in which hematopoiesis has been demonstrated in various locations in the normal mammalian embryo, chorionic villi,<sup>9</sup> the loose connective tissues,<sup>10</sup> breast tissue,<sup>11</sup> testes,<sup>1</sup> sole of the foot<sup>12</sup> and prostate.<sup>13</sup> In the lower vertebrates, the kidney, gonads, and intestinal tract are the site of hematopoiesis in adult animals.<sup>14</sup> In fish Scharrer<sup>15</sup> has described hematopoiesis in the meninges in the form of a definite organ of myeloid tissue in much the sense of mammalian marrow. Hematopoiesis has been seen in all of these areas in the present study in the embryonic rat.

It would appear from these studies in the lower vertebrates and in mammalian embryos that the mesenchyme at some time in embryonic or postnatal life is hematopoietic throughout most of its distribution. Furthermore, studies on embryonic tissues stimulated by infection,<sup>16</sup> toxins<sup>17</sup> and transplantation<sup>18</sup> have demonstrated that the potencies realized during normal embryonic life do not represent the sum total of mesenchymal potentialities of differentiation. By means of these experimental approaches, it has been shown that various localities, which, in embryonic life are not hematopoietic, or only slightly so, may become the site of active blood formation, or that the number of any cell type produced may be augmented or decreased.

Similarly, in syphilitic embryos and in embryos with erythroblastosis fetalis, active erythrocytogenesis may persist in the liver long past the usual time in normal human embryos. Miller<sup>19</sup> has described erythrocytogenesis in the heart and stomach of infants of prediabetic mothers.

However, in spite of this remarkable hematopoietic ability of the mesenchyme, there has been little evidence of any hematopoiesis in the central or peripheral nervous system in embryos or adults in any vertebrate. Gilmour,<sup>10</sup> in humans, described erythroblasts about the nerves in the meninges. This is very common in the rat embryo. Gutsell<sup>20</sup> illustrated an island of myeloid tissue in a peripheral nerve of a newborn infant. Collin and Baudo<sup>21</sup> and also Watrin<sup>22</sup> described erythropoiesis in the glandular (anterior?) lobe of the pituitary. However, since there were no illustrations and since the morphology of the cells they described was not characteristic of erythroblasts it is probable that they were describing cells with pyknotic nuclei in areas of spontaneous degeneration. The demonstration of hematopoiesis in the central and peripheral nervous system in this study, therefore, serves to substantiate still further the concept of the mesenchyme as having hematopoietic potentialities in all organs in whose structure it participates.

Since Rio-Hortega<sup>23</sup> separated the microglia from the other glial cells of the nervous system, there has been some controversy concerning the origin of these cells. Metz and Spatz<sup>24</sup> have traced them to spongioblasts and have summarized the evidence in favor of a neuro-epithelial origin. Rio-Hortega and a majority of workers have derived microglia from the mesenchyme. Since it is universally accepted that erythroblasts and hemocytoblasts are of connective tissue origin, the present study demonstrates that cells of connective tissue origin have entered into the neuro-epithelium in embryonic life. It is conceivable that some of these mesenchymal cells may serve as a source of microglia, the macrophages of the

nervous system, just as they serve as a source of histioid wandering cells, the macrophages of the embryonic connective tissues. This hypothesis receives further support because of the close relationship if not morphologic identity of the microglia of central nervous system and the macrophages of the connective tissues.<sup>11</sup>

The present study has failed to demonstrate penetration of the embryonic central nervous system by cells of mesenchymal origin except by migration along blood vessels. It is of interest that Von Santha and Juba,<sup>16</sup> employing Hortege silver carbonate impregnations of the nervous system in the embryonic rat, failed to demonstrate any mesenchymal invasion of the central nervous system other than by mesenchymal cells migrating along the blood vessels.

### CONCLUSIONS

1. Definitive erythropoiesis in the central and peripheral nervous system is a normal occurrence in the embryonic rat.

2. This is of significance because it is an illustration of the hematopoietic potency of the embryonic mesenchyme in an area where it has not been previously described.

3. It is of significance also because it presents unequivocal proof of the presence extravascularly of cells of connective tissue origin in the central nervous system. It is possible that some of these cells may serve as the precursors of the microglia.

4. There is no evidence that cells of connective tissue origin enter the central nervous system in any way except by migrating in along blood vessels.

### ACKNOWLEDGMENT

I wish to acknowledge the helpful criticisms of Dr. William Bloom of the Anatomy Department of the University of Chicago in whose laboratory the major part of this work was done.

### REFERENCES

1. BLOCK, M. An experimental analysis of hematopoiesis in the rat yolk sac. *Anat. Rec.* 96: 289, 1946.
2. MAXIMOW, A. Untersuchungen über Blut und Bindegewebe. I. Die frühesten Entwicklungsstadien der Blut und Bindegewebezellen beim Säugetierembryo bis zum Aufgang der Blutbildung in der Leber. *Arch. f. mikr. Anat.* 73: 444, 1909.
3. BLOOM, W. AND BARTELMER, G. Hematopoiesis in young human embryos. *Am. J. Anat.* 67: 21, 1941.
4. HELD, H. Über die Neuroglia marginalis der menschlichen Grosshirnrinde. *Monatsschr. f. Psych. u. Neurol. Ergänzungsheft* 26: 360, 1909.
5. SCHALTENBRAND, G. AND BAILEY, P. Die perivaskuläre Pialemembran des Gehirns. *J. f. Psychol. u. Neurol.* 31: 199, 1927-8.
6. MAXIMOW, A. Bindegewebe und Blutbildende Gewebe. *Handbuch der mikroskopischen Anatomie d. Menschen* Vol. II, p. 232. Wilhelm von Mollendorf, Berlin: Julius Springer, 1927.
7. ALFREY, S. Die embryonale Histogenese der Zellformen des lockeren Bindegewebes der Säugtiere. *Folia Hemat.* 30: 111, 1924.
8. SAXER, F. Über die Entwicklung und den Bau der normalen Lymphdrüsen und die Entstehung der ersten und weissen Blutkörperchen. *Anat. Hefte* 6: 347, 1896.
9. WISLOCKI, G. B. Hemopoiesis in the chorionic villi of the placenta of platyrrhine monkeys. *Anat. Rec.* 55: 349, 1943.
10. GILMOUR, T. Normal hemopoiesis in intra uterine and neonatal life. *J. Path. & Bact.* 52: 25, 1941.
11. GRUBER, G. Ueber die Milchdrüschenschwellung bei Neugeborenen (Zurück über extramedulläre Hämopoiesis). *Ztschr. f. Kinderh.* 30: 336, 1921.



# PATHOLOGY OF THE RUPTURED SPLEEN IN ACUTE VIVAX MALARIA

By JOSEPH M. LUBITZ, M.D.

**D**ESPITE the fact that malaria is a worldwide disease involving millions of individuals and considering the large number of cases seen in military service, spontaneous rupture of the spleen is actually a rare occurrence. Seventy-two cases were accumulated by Leighton prior to 1917 and 64 cases have been reported subsequent to this date.<sup>1</sup> Studies of the pathology of malaria are of importance in those parts of the world where malaria is prevalent. Previous pathologic descriptions of the spleen in acute tertian malaria in man have been sketchy. Furthermore, the mechanisms underlying the rupture have not been adequately studied. A pathologic description of the spleen in 4 cases of acute tertian malaria is therefore pertinent even though the incidence of malarial attacks and possibility of splenic rupture is rapidly decreasing. In 3 of the 4 cases under consideration, spontaneous rupture had occurred. The fourth case was that of a subcapsular hemorrhage, presumably just prior to rupture. All cases were successfully operated with splenectomy.

## PATHOLOGIC FINDINGS IN SPONTANEOUS RUPTURE IN ACUTE MALARIA

The pathology of the spleen in acute malaria has been described by Taliaferro and Mulligan<sup>2</sup> and by Ash and Spitz.<sup>3</sup> Of the 64 cases reviewed by us, only 29 included gross pathologic descriptions. These are summarized as follows. Grossly the spleen is enlarged with an average weight of 450 to 500 Gm. The consistency is soft, the color is reddish rather than slate gray as occurs in chronic malaria. Rupture may occur on any surface of the organ. It may be explosive or with multiple rents but in the majority of cases there is only one tear. The size of the tear varies from a small nick to 10.0 cm. A subcapsular hematoma is most probably the initial stage preliminary to the rupture.

In 16 of the 29 cases, microscopic descriptions were reported. These changes are essentially that of the pathologic changes of the spleen occurring in acute malaria in man and animal. Characteristic changes in the spleen are both cellular and vascular. Three distinctive features of the cellular changes are found:

*Cellular Changes* 1. *Phagocytic Activity* Macrophages proceed to remove by phagocytosis malarial pigment and disrupted erythrocytes. The heterophiles and lymphocytes play an insignificant part. The reticulum cells display the most prominent activities. These cells within the splenic cords become numerous, enlarged, predominating in the cellular picture of the spleen. Within the venous sinuses these macrophages can be seen engulfing not only the yellowish green pigment of the parasites, but also the red cells or hemoglobin derivatives. In the dilated splenic

Department of Pathology, Veterans Administration Center, Wood, Wisc., and Marquette University School of Medicine, Milwaukee, Wisc.

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within the widened splenic cords. They contained both blood pigment and malarial pigment granules. The endothelial cells were hyperplastic projecting into the lumen or desquamated into it. The dilated sinusoids contained desquamated endothelial cells few red cells scarce polymorphonuclear cells and large single and multinucleated macrophages containing iron and malarial pigment. The cellular mass lay close to the vessel wall often merging with the cord cells forming a thrombus. Veins and venules were frequently dilated forming pools containing the same cells that were seen in the sinusoids. In the subendothelial zone there was a layer of macrophages and monocytes containing pigment. In many portions of the spleen but particularly under the capsule not necessarily at the site of rupture there were large dilated veins and sinusoids which were thrombosed. The thrombus was comprised of a matrix of fibrinous material in which there were macrophages, hemolyzed and degenerating red cells and polymorphonuclear cells. Plasmodia could not be identified in the fixed tissue sections using the bismarck, toluidine and eosin or Romanowsky's stains. Scrapings from the spleen showed rare *P. vivax* within the red cells but not within the macrophages.

CASE II (Lap 90874) A 36 year old male who had served in New Guinea, Philippine Islands and Japan returning to the States one year previously was admitted to the hospital complaining of abdominal pain. He had had no attack while overseas and had been under atabrine prophylaxis. On physical examination the abdomen was tense. Tenderness was localized to the left upper quadrant. Dullness in the left lower lobe of the lung indicated pleural fluid. The admission red count was 2,600,000 hemoglobin 8.5 Gm. WBC 4850 temperature 101.4 degrees. X-ray examination indicated an enlarged spleen. *P. vivax* was found on blood smear and antimalarial treatment was started. It was not felt that splenic rupture had occurred but he was closely observed. On the ninth day he was operated. At surgery (Dr. Dwight Fishwick) a large amount of old dark blood was found in the peritoneal cavity. The spleen with a large subcapsular hematoma was found adherent to the diaphragm and walled off by omentum. Splenectomy was done. On the fifteenth day the lesion in the left lower lung was still present. The red count and temperature returned to normal and the patient was discharged. At the time of discharge blood count, temperature and white count were normal.

Gross (Fig. 1) The spleen weighed 400 Gm. measured 15.0 x 12.0 x 5.0 cm. Only at the hilus was the capsule intact. On the other surfaces the capsule was completely stripped from the splenic pulp by a large subcapsular blood clot measuring 9.0 x 7.0 x 1.0 cm. On the upper pole of the convex surface of the spleen there was an irregular rent measuring 2.0 cm. in diameter and 5.0 mm. in depth. On the opposite pole there were small irregular holes in the splenic tissue. Cut section revealed a soft purplish red pulp with a bulging parenchyma.

Microscopic The follicles were large with hyperplastic germinal centers in which there were few mitoses. The follicles were fairly well outlined against the red pulp. In the red pulp the splenic cords were thickened. reticulum cells were moderately hyperplastic. The sinusoids were poorly outlined. No malarial pigment was seen. Eosinophilic cells were present. The endothelial cells were moderately hyperplastic. In the trabecular veins there was a subintimal cushion of cells similar to those seen in the splenic cords. A thick layer of cells of similar type was found between the adventitia and muscularis. The vessels and sinusoids were often dilated and contained an admixture of cells with fibrin closely aligned to the wall. Small hemorrhages were seen deep under the capsule. No parasites could be identified in the tissues or the smears of the splenic pulp.

CASE III (Ric 84240) A 30 year old male was admitted to the hospital complaining of chills, fever, vomiting and malaise. He had served in New Guinea and had experienced several attacks of malaria in the past. The initial blood smear was positive for *P. vivax*, red blood cells 3,400,000, white blood count 12,600. During the afternoon of the day of admission he suddenly fainted. He recovered with pain in the epigastrium and then lapsed into shock. On physical examination there was guarding of the abdominal muscles and tenderness over the abdomen. The spleen was not felt. At surgery (Dr. John G. Slaney) the peritoneal cavity was found to be filled with bright red blood which was oozing from the spleen. The spleen was removed. Postoperatively there were no complications and the red count returned to normal.

Grossly the spleen weighed 255 Gm. and measured 14.0 x 8.0 x 4.5 cm. Over a distance of 9.5 x 7.0 cm. the capsule had been torn away from the surface. On the convex surface there was a small rent in

the splenic tissue measuring 50 mm. On section the splenic pulp was dark brown in color soft with prominent lymph follicles.

*Microscopic.* The follicles were hyperplastic with active germinal centers. The periphery of the follicles fused with the cellular red pulp. In the latter the splenic cords were thickened and the reticulum cells enlarged. The sinusoids were poorly defined and filled with macrophages. No malarial pigment granules or parasites were seen. The trabecular veins, venules and sinusoids were often dilated either empty or engorged with macrophages, red cells and polymorphonuclear cells. In the subintima and adventitia there was a layer of cells similar to those found in the cords. No thrombi were noted. Under the capsule there was a large hemorrhage which extended deep into the pulp. Smears from the splenic pulp were positive for *P. vivax* within the red cells. In one remote area in which the overlying capsule was intact numerous dilated venules were present filled with hemolyzed blood, red blood cells and white blood cells.



FIG. 1.—Case II. Spleen gross. Denudement of capsule and blood clot.

**CASE IV (And 90495).** A 22-year-old white male was admitted to the hospital with vague abdominal pain of sudden onset, chills and fever. He had served in the Southwest Pacific. While in Guadalcanal he had experienced several attacks of malaria. His last episode was in this country one year previously. His present illness began twelve hours before admission with chills and fever. As he had been a returned patient in the past, he took atabrine for what he considered to be another attack of malaria. On physical examination there was a splinting of the abdomen and pain in the upper abdomen on deep respiration. In the left lower lung breath sounds were slightly impaired. A ray showed slight opacity of the left lower lobe. Blood smears for malaria were repeatedly negative. While in the hospital pain gradually developed in the left upper quadrant, spreading to the lower half of the abdomen with increasing intensity. Admission temperature was 100.8 F., red count 4,950,000, hemoglobin 14.5 Gm., white count 12,000. During the next few days the patient became toxic, listless, and acutely ill. At no time was the spleen palpable. Since the pain was not fully localized and smears were repeatedly negative for parasites,



FIG 2.—Case IV Spleen gross Indentation of capsule underneath which there is a subcapsular hematoma



FIG 3.—Case IV Spleen microphotograph (low power) Showing well defined subcapsular hematoma

th diagnosis was held in abeyance and he was further observed. However a diagnosis of ruptured malarial spleen being suspected it was considered advisable to explore him on the sixth day following hospitalization. At surgery (Dr. James Sullivan) the spleen was found to be about double its size. There was perisplenitis but no hemorrhages were seen. The spleen was removed.

*Gross* (Fig. 2) The spleen weighed 300 Gm. and measured  $13.0 \times 9.0 \times 4.0$  cm. The capsule was dull and covered with fibrinous shreds. No rent or hemorrhage was seen on external examination. On cut section, on the convex surface a localized subcapsular hemorrhage measuring 5.0 mm. was found under a small indentation of the capsule. The parenchyma was brownish red in color and dripping. The follicles were small but prominent.

*Microscopic.* The capsule was slightly thickened, the serosal cells hyperplastic and covered with fibrin. The area seen grossly as hemorrhage was found to be well defined (fig. 3). Nearby were smaller diffuse hemorrhages. All sinusoids under the capsule were severely engorged with blood. An occa-

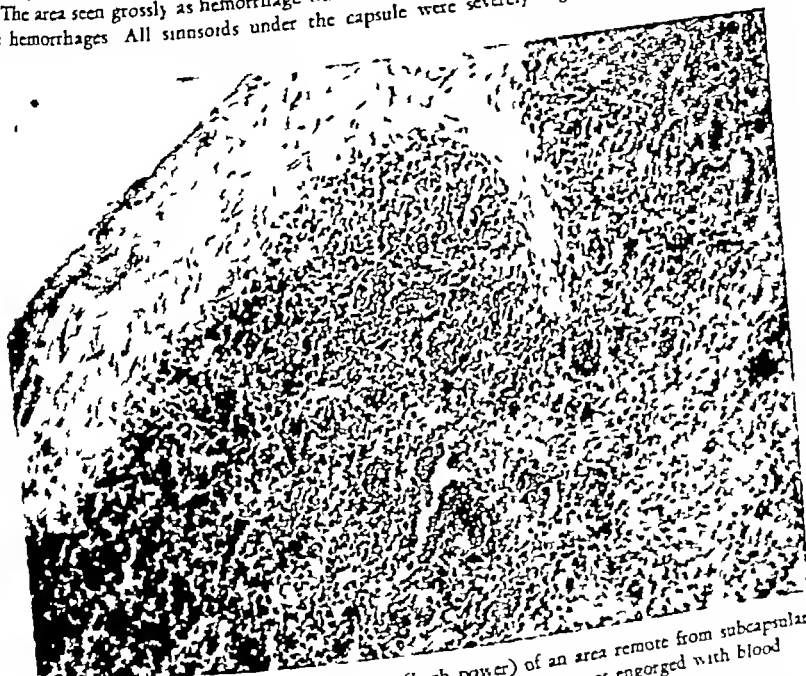


Fig. 4—Case IV. Spleen, microphotograph (high power) of an area remote from subcapsular hematoma. Fibrinous perisplenitis, cellular hyperplasia and dilated sinusoids engorged with blood.

sional dilated sinusoid or vein was filled with erythrocytes, particularly under the capsule at sites remote from the hematoma (fig. 4). The follicles were prominent with active germinal centers showing large reticulum cells with mitoses. Nuclear fragments were not seen. The margin of the follicles merged imperceptibly into the cellular red pulp. The thickened splenic cords contained numerous reticulum cells which were somewhat elongated but not as vesicular as found in previous cases. The endothelial cells were hyperplastic and rounded. The sinusoids were collapsed and devoid of erythrocytes. They contained however numerous large pale cells resembling reticulum cells, many of which contained occasional polymorphonuclear cells and rarely multinucleated cells.

#### Discussion

A review of the literature indicates that malaria is the most common single cause of spontaneous rupture of the spleen. Smith and Custer<sup>1</sup> found no cases of

as Spleen, spontaneous rupture, in the records of the Army Institute of Pathology. Of these, 22 were recurrent malaria, 7 infectious mononucleosis, 5 Banti's disease, 2 leukemia, 3 torsion, and 5 cause unknown. Of the 64 reported cases reviewed in the literature up to 1946,<sup>1</sup> 25 were in naturally acquired malaria and 39 in inoculation malaria. The incidence of spontaneous rupture in induced malaria is higher than in acquired malaria. The age of the patient, the repeated paroxysms of malaria without specific therapy, and the failure to recognize the symptoms of rupture probably account for the higher mortality from rupture of the spleen in the induced group. In the chronic stage of malaria, rupture of the spleen is rarely described in this climate. However, rupture by minimal trauma in malarious zones is frequent. *P. vivax* is the most common infecting agent, although other species of plasmodia have been described.

The mechanism of rupture is probably three-fold: (1) increase of intrasplenic tension due to cellular hyperplasia and engorgement, (2) compression by abdominal musculature, (3) local lesions due to vascular occlusion. Rigdon's hypothesis of obstruction of the vessels by hyperplasia is supported by our findings. His findings

TABLE 1—*Histopathology of Spleen in Acute Malaria*

Case		Cellular infiltration		Sinuses			Reticulum hyperplasia	Active germinal centers	Dilated veins and venules	Thrombosis	Infarction	Subcapsular hemorrhages
		Veins	Capsule	Hyperplastic endothelium	Obstruction	Dilatation						
1	11948	+	—	+	+	+	+	+	+	+	+	+
2	90874	+	—	+	+	+	+	+	+	—	—	+
3	84240	+	—	+	+	+	+	+	+	—	—	+
4	90495	+	—	+	+	+	+	+	+	—	—	+

in experimental animals are identical with those observed in humans. He believes that reticular and endothelial hyperplasia obstructs venules and sinuses resulting in thrombosis and infarction which cause interstitial and subcapsular hemorrhages. Subcapsular bleeding strips the capsule resulting in further hemorrhage with distention of the capsule and final rupture. In all four cases (table 1) a common finding was the presence of dilated sinuses and hemorrhages immediately under the capsule. Although infarctions have been frequently described in animals,<sup>6</sup> in only 1 of our 4 cases was infarction found. It alone, therefore, cannot be the sole cause of rupture.

Subintimal and periaventricular leukopoiesis is of special interest and is a striking finding. This appears to be a reversion to embryonal potency. Such leukopoiesis also occurs in cases of infectious mononucleosis.<sup>8</sup> One such case described by Sullivan and Wassermann<sup>7</sup> was available for study. It, too, showed extreme hyperplasia but the hyperplastic cells were typically infectious mononucleosis cells rather than the histiocytic cells seen in malaria. Jaffé and others<sup>8</sup> have described similar changes in acute leukemia with leukemic cells in the walls of the vessels. Smith and Custer discuss the histopathologic changes of ruptured spleen in infectious mono-

nucleosis. They found changes which closely resemble those seen in malaria, such as blurred pattern representing general hyperplasia, cellular subintimal infiltration in arteries, veins, trabeculae and capsule. It differs from the malarial cases which we have studied in failure of leukopoiesis to occur in arteries, absence of dilated veins, thrombosis or infarction in infectious mononucleosis. Thus, from the reported cases of infectious mononucleosis and from failure to find infarction and thrombosis in three of the four cases studied, it can be concluded that these vascular changes alone are not necessary for rupture to occur. Smith and Custer noted dissolution of the capsule and trabeculae due to cellular infiltration and edema. They believe that rupture was due to a rapidly expanding organ with damage of the enveloping framework. From our studies it would seem that dilated sinuses and small subcapsular hemorrhages occurring in the malarial spleen are important factors, initiating the hematoma. We agree with Smith and Custer that a subcapsular hematoma must occur prior to rupture. The subcapsular hemorrhage described in Case IV probably represents this stage. Under sufficient tension due to the general cellularity and frequently by additional pressure due to minimal trauma, this hemorrhage may rupture through the capsule.

Referred pain from the diaphragm to the shoulder via the phrenic nerve has been described in 6 cases of ruptured malarial spleen. It has also been commented on in ruptured spleen due to infectious mononucleosis. However, perhaps more significant is the presence of irritation of the left lower lobe of the lung. This elicits impairment of breath sounds, dullness, and opacity by x-ray examination. In Cases II and IV pleural fluid was diagnosed before surgery. In the previously cited case of infectious mononucleosis by Sullivan and Wassermann,<sup>7</sup> pleural fluid was also noted before surgical intervention. In 3 cases of spontaneous rupture of the spleen reported by Littenfield,<sup>9</sup> pulmonary complications developed in the left lung field. However, they noted this only following surgery. This pleural irritation is probably due to perisplenitis with transmigration of toxic irritants through the diaphragm. In cases in which a diagnostic problem is presented, this sign may be of value.

#### SUMMARY

1. Opportunity to study the pathologic changes in ruptured spleen of acute vivax malaria was afforded by splenectomy.
2. Subcapsular hematoma precedes rupture.
3. The capsular tear is an obvious consequence of the changes in the spleen occurring in acute malaria. Small hemorrhages occur in the vicinity of the capsule or deep in the tissues.
4. A rapidly enlarging spleen with underlying vascular alteration predisposes to rupture. Minimal trauma may be a precipitating element.
5. Diffuse cellular hyperplasia, subintimal and adventitial leukopoiesis, dilated sinuses and occasional thrombosis and infarction constitute the characteristic pattern in malaria.
6. Changes in the left lung base may serve as a diagnostic aid in cases in which the diagnosis of splenic rupture is considered.

## REFERENCES

- <sup>1</sup> HERSHEY F B AND LUBITZ J M Spontaneous rupture of the malarial spleen *Ann Surg* 127 40, 1948
- <sup>2</sup> TALIAFERRO W H AND MULLIGAN H W Histopathology of Malaria with Special Reference to the Function and Origin of the Macrophages in Defence *Ind Med Res Memoirs* No 29 May 1947
- <sup>3</sup> ASH J E AND SPITZ S Malaria Pathology of Tropical Diseases 206 1945
- <sup>4</sup> RIODON R H A consideration of the mechanism of splenic infarcts in malaria *Am J Trop Med* 24 349 1944
- <sup>5</sup> SMITH E B AND CUSTER R P Rupture of the spleen in infectious mononucleosis *Blood* 1 317, 1946
- <sup>6</sup> HEWETT R Splenic enlargement and infarction in canaries infected with a virulent strain of *Plasmodium cathemerium* *Am J Hyg* 30 49 1939
- <sup>7</sup> SULLIVAN J AND WASSERMANN S Spontaneous rupture of the spleen due to infectious mononucleosis *J A M A* 134 144 1947
- <sup>8</sup> JAFFE R H Histologic changes of the spleen in cases of leukemia *Arch Path* 19 647 1935
- <sup>9</sup> LITTENFIELD J B Spontaneous rupture of the spleen *Surg, Gynec & Obst* 82 207 1946

# ABSTRACTS

JOSEPH F. ROSS M.D., Editor

## ABSTRACTERS

HELEN W. BELDING, M.D. Winston Salem N.C.	OLIVER P. JONES Ph.D. Buffalo
SHEILA T. CALLENDER M.D. Oxford England	CONRAD MAIER M.D. Zurich Switzerland
GEORGE E. CARTWRIGHT M.D. Salt Lake City	MILOS NETOUSEK M.D. Prague, Czechoslovakia
ROGER C. CRAFT Ph.D. Boston	JEAN P. SOULIER M.D. Paris
CHARLES P. EMERSON M.D. Boston	RAMON M. SUAREZ M.D. San Juan Puerto Rico
SOLOMON ESTREN M.D. New York	WILLIAM N. VALENTINE M.D. Pacific Palisades Cal
CLEMENT A. FINCH, M.D. Seattle	JAN WALDENSTROM M.D. Upsala Sweden

## HEMATOPOIETIC TISSUES

EFFECTS OF FOLIC ACID DEFICIENCY AND A FOLIC ACID ANTAGONIST ON CHICKS. *E. Wolf* From the Research Department Lederle Laboratories Pearl River N. Y. Arch. Path. 46 559-566 1948

Rapidly growing subjects like newly hatched chicks readily lend themselves for such purposes as the study of nutritional disturbances. It was for this reason that they were selected for experiments in folic acid deficiencies. About 200 one day old New Hampshire Red chicks were divided equally into the following six dietary groups: control; folic acid free diet; folic acid free diet plus folic acid by injection; regular diet plus 4 aminopteroylaspartic acid in the diet; regular diet plus 4 aminopteroylaspartic acid by injection; and folic acid free diet for ten days followed by same diet supplemented with a daily intraperitoneal injection of 0.1 mg. of folic acid. Changes in the folic acid-deficient birds and those in the birds treated with antagonist were essentially the same. Of the organs involved, the bone marrow and bowel showed the greatest departure from normal. The ultimate picture of the marrow was that of a severe aplasia with a myxomatous appearance of the connective tissue. In the distal third of the small bowel there was an atrophy of the mucosa with the appearance of retention cysts and fibrous elements of the stroma. Folic acid will prevent these changes when injected into folic acid-deficient animals. O. P. J.

HISTOPATHOLOGIC OBSERVATIONS IN CASES OF HODGKIN'S DISEASE TREATED WITH NITROGEN MUSTARD. *V. H. Cornell and A. S. Blau* From the Laboratory Service, Walter Reed Hospital, Washington D. C. Am. J. Path. 25 233-237 1949

Clinical response to the use of intravenous nitrogen mustard in diseases of the lymphatic system has been reported by various authors since 1944. In order to determine possible differences in lymph node architecture before and after therapy, 17 cases from a series of 55 were selected for study. The most surprising thing was the absence of a single criterion that could be said to exist in any two treated cases. The authors point out that it is difficult to rationalize the use of a drug which primarily attacks the lymphocytes in the treatment of a disease generally considered to be one of the reticuloendothelium. O. P. J.

EFFECT OF CONTINUOUS RADIATION ON CHICK EMBRYOS AND DEVELOPING CHICK. II. BONE MARROW LYMPHOID TISSUE AND PERIPHERAL BLOOD. *S. Warren and J. F. J. Dixon* From the Laboratory of Pathology of the Harvard Cancer Commission, Boston, Mass. Radiology 53 669-670 1949

By means of radioactive phosphorus ( $P^{32}$ ) the effects of lethal and sublethal irradiation on the bone marrow were studied in chick embryos and developing chicks. There was an overall retardation of the growth of the birds; a marked retardation of bone growth and especial sensitivity was noted in the skeletal system and ovaries. In the present paper the effects on bone marrow, lymphoid tissue and peripheral blood are recorded.

In the bone marrow the effects were the result of the heavy irradiation of marrow cells and the concentration of  $P^{32}$  within the bones. Sublethal irradiation resulted in a marked retardation of the growth of the birds.



poietic cells and a reduction of mitotic activity so that pancytopenia developed maximally in 1 to 2 weeks. In 2 to 3 weeks recovery began with an increase in mitotic activity and later maturation of the blood cells. When irradiation was lethal there was a complete halt of mitotic activity and of maturation within 2 to 3 days after use of  $P^{32}$  with resultant hypoplasia of the marrow, fatty degeneration within the marrow and progressive pancytopenia with death in 2 weeks. In both lethal and sublethal actions the reticulo-endothelial cells were little affected and tended to give rise to primitive blood cells during recovery.

In lymphoid tissue  $P^{32}$  caused suppression of mitoses and a thinning out of the lymphocytes. This response was followed rapidly by prompt and marked regeneration of lymphoid tissue with recovery being complete in most instances. Regeneration was excessive in amount.

In the peripheral blood the lymphocytes fell rapidly and with recovery returned fairly rapidly to normal. The granulocytes fell more slowly; an agranulocytosis was present within 2 weeks and the recovery phase was slower than for the other blood cells. The red cells fell quite slowly following a lag period and recovery was quite rapid although slower than the return of the lymphocyte level to normal. S. E.

**INFLUENCE OF LOCAL ACIDIFICATION OF TISSUE BORNEING CANCEROUS GROWTHS WITH SPECIAL REFERENCE TO THE EOSINOPHIL, THE PANETH CELL AND THE ACIDOPHILIC PLASMA CELL.** C. E. Black and R. S. Ogle

From Department of Pathology, Sparrow Hospital, Lansing, Mich. Arch. Path. 46: 107-118, 1948

Eosinophils, Paneth cells and acidophilic plasma cells are usually more numerous in the lamina propria of the small intestine than they are in either the stomach or large bowel. Collections of acidophilic cells which are usually in the vicinity of cancerous growths seem to be a defensive response of the host. Acidophilic cells are seldom the source of primary neoplasm. There may be some relationship between the presence of these cells in the small intestine and appendix and the infrequency of carcinoma or tendency slowly to metastasize there.

O. P. J.

**THE RATE OF MITOTIC ACTIVITY IN THE LYMPHOID ORGANS OF THE RAT.** E. Andreassen and S. Christensen

From the Department of Anatomy, Faculty of Medicine, University of Copenhagen, Denmark. Anat. Rec. 103: 401-412, 1949

In studies of mitotic activity with sectioned material of lymphoid tissue the results have not been wholly reliable because of the irregular and capricious distribution of dividing cells. In an effort to determine the proportion of dividing cells visible in a population, suspensions of cell nuclei were made by treating finely divided tissue with 5 per cent citric acid for one half to one hour. After a series of centrifugations, staining with galloxyaniline, resuspensions in absolute alcohol and finally benzyl benzoate, ten thousand nuclei were counted in a Bürker-Türk counting chamber. Mitotic phases before the disappearance of the nuclear membrane and after reconstruction of the nuclear membrane in telophase were rarely seen. Hence the counts involved phases between these two extremes. Thymic lymph nodes and spleens were examined from rats of three different age groups: 1 c, 1 month, 3 months and 8-12 months old. The number of mitotic figures per thousand nondividing nuclei was consistently higher in the thymus than in either lymph nodes or spleen. There was a decrease with age which was more noticeable in lymph nodes and spleen. Additional experiments were conducted to determine the source of lymphocytes in rats during restitution after starvation. The thymus reaches its normal mitotic activity 3-4 days after restitution while lymph nodes and spleen show no deviation from normal either at the end or during restitution. O. P. J.

**CHANGES IN THE CAPSULE OF THE LYMPH NODE IN EXPERIMENTAL HYPERPLASIA.** W. J. Furness

From the Department of Anatomy, University of Illinois College of Medicine, Chicago, Ill. Arch. Path. 47: 273-282, 1949

Lymph node hyperplasia is a common occurrence in some blood dyscrasias and under certain experimental conditions. Attention has been generally directed toward structural changes of the lymphatic tissue and not of the capsule. In order to study the latter, experimental hyperplasia was produced in hamsters and rats by injecting Eberthella typhosus vaccine. Hyperplastic mediastinal and bronchial lymph nodes from calves with acute bronchopneumonia were obtained from the slaughter house. In

hamsters and rats hyperplastic nodes had a portion of the cortex extending through areas deficient in capsule because of a previous rupture. Maximal peripheral expansion of these nodes was observed in the hilar region. The degree of extracapsular migration of lymphatic tissue into the perinodal areolar tissue increased with the length of the postinjection period. In the hyperplastic calf lymph node the capsule did not rupture because of its thickness.

O P J

THE HISTOLOGICAL PREPARATION OF BONE MARROW PARTICLES: UTERINE CURETTINGS AND OTHER SMALL TISSUE FRAGMENTS. A C P Campbell From the Department of Pathology, Edinburgh University, Edinburgh, Scotland. *J Path & Bact* 60: 633-634, 1948.

The author uses agar for embedding particles after fixation. This procedure reduces the forceps trauma and permits a single block easily to be carried through subsequent steps.

O P J

## ERYTHROCYTE PHYSIOLOGY

INFLUENCE OF ENDOCRINE FACTORS ON HEMOPOIESIS IN THE ADULT FROG, *RANA PIPIENS*. E T Bossak, A S Gordin, and H A Charipper. From Department of Biology, Washington Square College, New York University, New York, N Y. *J Exper Zool* 109: 13-31, 1948.

The hormonal control of erythropoiesis has been investigated in the mammal and bird but not in the amphibia. The adult frog normally has hypoplastic marrow during hibernation so that perhaps the posthibernation erythropoiesis may be the result of participation by the endocrine system. Adult frogs of both sexes were kept in refrigerators at a constant temperature of 4-6°C before and during the experiment. Four groups of test animals received 20 injections of sesame oil, thyroxine, testosterone propionate, and estradiol benzoate respectively. The animals were sacrificed 45 days after start of injections. The results indicate that there is some relationship between the endocrine system and hemopoiesis but further study is necessary in order to understand the mechanism.

O P J

ON THE NATURE AND SIGNIFICANCE OF STIPPLING IN LEAD POISONING WITH REFERENCE TO THE EFFECT OF SPLENECTOMY. A J S McFadzean and L J Davis. From the University Department of Medicine, Royal Infirmary, Glasgow, Scotland. *Quart J Med* 18: 57-72, 1949.

In both human patients and in guinea pigs with lead intoxication stippling is demonstrable in the bone marrow in normoblasts as well as in the non-nucleated erythrocyte in marrow and peripheral blood. A positive reaction for iron is exhibited by a variable proportion of the granules and there is often associated evidence of defective hemoglobinization in the affected cells. It is of interest that in the experimental guinea pigs employed in these studies splenectomy ameliorated or prevented the anemia of lead intoxication as well as increased the relative proportion of stippled cells in the circulation and the frequency of a positive reaction for iron in the granules. The suggestion is made that lead interferes with hemoglobin synthesis with partial failure in the incorporation of iron into the protoporphyrin ring. Removal of the more defective cells by the spleen could thus explain the hemolytic component of lead intoxication and the beneficial effect of splenectomy on experimental lead-induced anemia. It is possible that a similar mechanism may explain the beneficial results of splenectomy in a peculiar acquired hemolytic anemia previously described by the authors in which iron-containing inclusion bodies were demonstrated in the erythrocytes (*Glasgow M J* 28: 237, 1947).

W N V

THE RATE OF LOSS OF POTASSIUM FROM HUMAN RED CELLS IN SYSTEMS TO WHICH LYSINS HAVE NOT BEEN ADDED. E Ponder. From the Nassau Hospital, Mineola, Long Island, New York. *J Gen Physiol* 35: 461-479, 1949.

In previous studies on the human red cell the loss of K increases with time until the K concentration inside the cell is approximately the same as that in the medium outside in systems containing lysins. When no lysin has been added the losses are rapid at first and they tend to slow down so that a new steady state remote from equilibrium is reached. The present experiments concern four such kinds of

systems (1) Washed red cells in saline at 4 C (2) washed red cells in saline at 25 C (3) washed red cells in saline at 37 C and (4) washed red cells in systems at 4 C 25 C, and 37 C containing hypotonic saline glucose or a number of other substances. In order to prevent bacterial contamination and thereby possibly introduce a hemolysin, all of the experiments were conducted under aseptic conditions based on the method used by Osgood for marrow culture

OPJ

THE RELATIVE RATE OF PENETRATION OF THE LOWER SATURATED MONOCARBOXYLIC ACIDS INTO MAMMALIAN ERYTHROCYTES *J W Grinn* From the Physiological Laboratory, Princeton University Princeton N J *J Cell & Comp Physiol* 33 247-266 1949

Since the work of Overton it has been widely accepted that compounds soluble in lipid solvents penetrate cells by reason of their solubility in the lipids of the cell surface. A new method was devised to measure the relative rate of penetration of the lower fatty acids into mammalian erythrocytes. This method, a chemical one, depends upon the fact that oxyhemoglobin loses some of its oxygen when placed in an environment containing an increased hydrogen ion concentration. The relative rates of penetration of the fatty acids investigated were found to be (a) for beef cells: caprylic < heptylic < caproic = valeric = butyric > propionic > acetic > formic (b) for human cells: caprylic = heptylic < caproic < valeric > butyric > propionic > acetic > formic. The rates of hemolysis by these acids were determined and found to be (a) for beef cells: caprylic > heptylic > caproic > valeric < butyric > propionic > acetic < formic (b) for human cells: caprylic > heptylic > caproic < valeric < butyric > propionic > acetic < formic.

OPJ

CRYPTOGENIC METABOLISM OF ERYTHROCYTES *L Heilmeyer and Th Eilers* From the Medical Clinic of the University of Heidelberg Schweiz med Wschr 78 975-76 1948

In an investigation of the metabolism of hemoglobin in pernicious anemia, the quantity of eliminated urobilin was found to be essentially higher than the reticulocyte level would indicate. By adding Giemsa stain for 8-12 hours in the refrigerator to the usual staining of reticulocytes with brilliant cresyl blue, pathologic reticulocytes of crescent form and containing many vacuoles are brought in evidence. These forms are not visible when the smears are stained in the usual way. They are more frequently found in smears from the spleen than in the peripheral blood. These reticulocytes are believed to be destroyed particularly fast within 24 to 48 hours. Even if they do not contain the final hemoglobin, they possibly represent prestages causing the increased metabolism of urobilin.

C.M

ESTIMATION OF RELATIVE CORPUSCLE AND SERUM VOLUMES IN BLOOD BY VARIOUS APPLICATIONS OF THE DILUTION PRINCIPLE. *P L McLain and C H W Rube* From the Departments of Physiology and Pharmacology, University of Pittsburgh School of Medicine, Pittsburgh Pa. *Am J Physiol* 156 12-18 1949

It has long been recognized that centrifugal methods do not completely separate corpuscles from plasma. Attempts to measure the amount of fluid left in the sediment have not given constant results. These measurements have usually been based on some application of the principle of serum or plasma dilution. The present study was done on whole beef blood. Relative corpuscle and serum volumes were estimated by 12 different applications of the serum dilution principle. These results were then compared with those obtained by centrifugalization. Mean differences between centrifugal and dilution results varied with the method from  $2.0 \pm 1.2$  to  $17.9 \pm 10.8$  per cent of the packed cell volume. Most dilution procedures are not well adapted to accurate serum volume estimates. The authors conclude that correction of conventional hematocrit results by a constant factor based on dilution methods is not justified.

R.C.C.

## BLOOD GROUPS

MOTHER-CHILD ABO INCOMPATIBILITY. A RELATION OF SECRETOR STATUS TO MENTAL DEFICIENCY. *H Yarnit and R Lieberman* From the Southbury Training School and the Department of Pediatrics Yale Medical School, Southbury Conn. *Am J Dis Child* 76 176-183 1948

Theoretically fetal damage from ABO maternal isoimmunization may result when the concentration of A or B factor in the child's body fluids is not sufficiently great to neutralize A or B maternal agglutinins.

Two hundred and eighty mentally deficient children were studied to determine the incidence of mother-child ABO incompatibility and the secretor status of the child. One hundred and fifty-seven of these had clinically defined mental deficiencies such as mongolism and were used as controls whereas the remaining 123 were classified as undifferentiated congenital amentia of unknown origin. Evidence is presented to show the unreliability of the study of a single undiluted specimen of saliva or gastric juice and in this study secretor status was determined only after both gastric juice and saliva were simultaneously examined and titrated.

There were 20 children who showed ABO maternal incompatibility and a nonsecretor status. The incidence of incompatible nonsecretors was greater in the group of undifferentiated mental deficiency (13 per cent) than in the control group (3 per cent) and it is suggested that ABO isoimmunization may be an etiologic factor in a small proportion of children classified as undifferentiated congenital deficiencies.

Even if confirmatory evidence were forthcoming it would be necessary to conclude, from the significant clinical differences pointed out by the authors, that the mechanisms responsible for cerebral damage in ABO and Rh incompatibility are not similar. One wonders because of the variable nature of secretor status whether the concentration of A or B factor in body fluids later in life is a valid measurement of that present in the prenatal and neonatal states.

H W B

IMMUNIZATION OF BLOOD DONORS WITH SALIVA V. Bzdorsky From the State Health Institute, Prague, Czechoslovakia. *Čas. lékař. Čes.* 87: 725, 1948.

Immunization of blood donors with diluted saliva of A and B secretors performed according to Wiener's proposal increased the titer of hemagglutinins in 80 per cent of cases; the hemagglutinin titer rose on an average eight times and was constant even after nine months. Better results of immunization were obtained in persons under the age of 45.

M N

SECTION 6. MICROBIOLOGY (a) THE RH FACTOR—GENERAL SIGNIFICANCE AND METHODS OF STUDY. P. Levine From the Ortho Research Foundation, Raritan, N. J. (b) RECENT VIEWS ON THE GENETICS OF THE RH BLOOD FACTORS. H. H. Strandskov From the University of Chicago, Chicago, Ill. (c) MEDICOLEGAL ASPECTS OF THE RH BLOOD TYPES. A. S. Wiener From the Office of the Chief Medical Examiner of New York City, New York, N. Y. (d) APPRAISAL OF THE CLINICAL ASPECTS OF THE RH FACTOR. P. Vogel From the Mount Sinai Hospital and Department of Health, New York City, New York, N. Y. *Bull. New York Acad. Med.* 25: 244-263, 1949.

These four papers deal briefly with the current views on the genetics of the Rh blood factors, the medical application of our knowledge of the several known blood types, the methods of detection of immunization by the Rh factor, and the management of erythroblastosis fetalis.

The author of the second paper proposes the use of the basic locus symbol Rh with the letters CDE as superscripts to clarify further the nomenclature introduced by Fisher and Race. The two gene hypotheses to account for inheritance of Rh blood types (e.g., the S allele hypothesis and the two-allele hypothesis) are discussed by the same author from the point of view of serologic evidence, gene results, and evidence obtained from gene-genotypic and phenotypic frequency analyses.

H W B

BLOOD TRANSFUSIONS AND THE RH FACTOR. P. G. Hattersley From the Department of Medicine, University School of Medicine, San Francisco, Calif. *California Med.* 37: 325-329, 1949.

Two cases are reported: one of fatal erythroblastosis in first and second pregnancies of a Rh-negative patient previously immunized by transfusions; the other of a major hemolytic reaction in a Rh-negative male patient previously immunized by a single transfusion of Rh-positive blood. The author further points up the necessity of administering Rh-negative blood only to Rh-negative patients. A second reaction was notable in that while laboratory evidence of complete hemolysis was absent within two hours of transfusion, was dramatic clinical symptom of a major hemolytic reaction.

# BOOK REVIEWS

*An Atlas of Bone Marrow Pathology* By M. C. G. ISRAEL'S New York Grune & Stratton 1948 \$6 50 79 pages

This small book of 79 pages centers around twelve color plates of marrow cells seven plates being composed of groups of single cells and five plates made up of various abnormal conditions. The descriptions of cell morphology are unusually good with the simplicity and lucidity we have come to associate with Israel's work. The black and white line drawings and the color plates are accurately done, although the latter lack brilliance. The Atlas can be recommended as a primer in the study of marrow puncture techniques.

WILLIAM DAMESHEK

*Hematology* By CYRUS C. STURGIS Springfield Ill C. C. Thomas Company 1949 \$12 50

*Hematology* ed. 2. By WILLIS M. FOWLER New York P. B. Hoeber Inc. 1949 \$8 50

Recent American texts of hematology are rapidly filling the large gap formerly existing in this country between the wide interest in the subject and the available number of standard works. Sturgis' large tome is new. Fowler's which is slanted frankly for students and practitioners is presented as a revised second edition.

Sturgis has an interesting preface in which he presents some of his views about hematology as a specialty the importance of some knowledge of the historical aspects of a given subject and of a carefully edited and comprehensive bibliography. The book begins in a rather unorthodox way in that the anemias are dealt with first. There is no attempt to delve into such matters as blood formation histology of blood cells etc. that are customarily discussed in the first few chapters of a hematologic text. Perhaps this is justified for a book of this type since many practitioners would probably skip such sections and if they were sufficiently interested could consult some more complete reference work on the subject. The book, from this viewpoint is eminently practical. The comprehensive historical discussions are of unusual interest and not to be found in any other similar work. They are worth the price of the book alone. The bibliographic references 1830 in number are presented numerically at the bottom of the pages referring to the publications in question and are repeated in more than fifty pages of alphabetically arranged bibliography at the end of the book.

There can be no question but that Sturgis' work is a valuable addition to the hematologic literature although some might criticize the methodology of presentation and the lack of both cytologic and physiologic approach to the various diseases.

Fowler's revised book is a distinct improvement over the first edition. It is more carefully written. It reflects adequately the advances made in the past few years and should serve as a primer in hematology.

WILLIAM DAMESHEK

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# Contributors to this Issue

## WILLIAM C. BOYD, Ph D

Professor of Immunochemistry, Department of Biochemistry Boston University School of Medicine Boston Mass

## GEORGE T. BRADFORD, M D

Baltimore Rh Laboratory Fellow in Medicine Department of Medicine University of Maryland School of Medicine Baltimore Md

## WILLIAM H. CROSBY (MAJOR, MC, AUS)

Resident in Internal Medicine Brooke General Hospital Fort Sam Houston Texas Present address Blood Laboratory Pratt Diagnostic Hospital Boston Mass

## J. V. DACIE, M B, M R C P, LONDON

Reader in Hematology Department of Pathology Postgraduate Medical School of London London England

## ROSE T. DUANE, A B

Technician Department of Medicine Stanford University School of Medicine San Francisco, Calif

## D. C. ELTZHOLTZ, CPHM, USN

Hematology Facility Naval Medical Research Institute Bethesda Md

## DONALD M. ERVIN, M D

Fellow in Medicine The University of Rochester School of Medicine and Dentistry Rochester N Y

## ROBERT S. EVANS, M D

Associate Professor of Medicine Stanford University School of Medicine San Francisco Calif

## JOSEPH A. GUILBEAU, JR, M D

Baltimore Rh Laboratory Fellow in Obstetrics Department of Obstetrics University of Maryland School of Medicine Baltimore Md

## ELSA F. JAHN, B S

Research Associate and Technician in Charge Baltimore Rh Laboratory Baltimore Md

## B. S. KLINE, M D

Chief Laboratory Department Mount Sinai Hospital Assistant Professor of Pathology Western Reserve University Cleveland Ohio

## WILLIAM J. KUHN, M D

U S Public Health Service Fellow in Hematology Department of Medicine University of Utah College of Medicine and Salt Lake County General Hospital Salt Lake City Utah

## F. DOUGLAS LAWRASON, M D

James Hudson Brown Fellow Department of Internal Medicine Yale University School of Medicine New Haven Conn

## PHILIP LEVINE, M D

Ortho Research Foundation Raritan N J

## ERIC PONDER, M D, D Sc.

Nassau Hospital Mineola, N Y

## ROSE M. REGUERA, B S

Research Assistant Department of Biochemistry Boston University School of Medicine Boston Mass

## HARRY J. SACKS (CAPTAIN, MC, AUS)

Present address Resident in Pathology University of Indiana Medical School Indianapolis Ind

## MILTON S. SACKS, M D

Associate Professor of Medicine University of Maryland School of Medicine Director of Department of Clinical Pathology University of Maryland School of Medicine and University Hospital, Director Baltimore Rh Laboratory Baltimore Md

## P. K. SCHORE, CPHM, USN

Hematology Facility Naval Medical Research Institute Bethesda Md

## C. R. SIPE, CPHM, USN

Hematology Facility Naval Medical Research Institute Bethesda Md

## THEODORE F. VAN ZANDT, A B

Student Fellow in Pathology The University of Rochester School of Medicine and Dentistry Rochester N Y

## LAWRENCE E. YOUNG, M D

Assistant Professor of Medicine The University of Rochester School of Medicine and Dentistry Assistant Physician Strong Memorial Hospital Rochester N Y

## CHARLES L. YUILE, M D

Associate Professor of Pathology The University of Rochester School of Medicine and Dentistry Pathologist Strong Memorial Hospital Rochester N Y

# BLOOD

## *The Journal of Hematology*

VOL IV, NO 11

NOVEMBER, 1949

### DIAGNOSIS AND MECHANISM OF HEMOLYSIS IN CHRONIC HEMOLYTIC ANEMIA WITH NOCTURNAL HEMOGLOBINURIA

By J V DACIE, M B , M R C P , LONDON

CHRONIC hemolytic anemia with nocturnal hemoglobinuria or nocturnal hemoglobinuria is an uncommon but most interesting form of chronic hemolytic anemia. Despite major contributions to the understanding of the mechanism of hemolysis made by Ham<sup>1, 2</sup> and Ham and Dingle<sup>3</sup> in 1937 and 1939, and by Dacie, Israëls and Wilkinson<sup>4</sup> and Jordan,<sup>5</sup> working independently at about the same time, the essential basis of the disease is still a mystery. The above mentioned workers showed that the primary abnormality resided in the patient's own erythrocytes, a proportion of which would undergo hemolysis when suspended in vitro in fresh unheated serum obtained either from the patient or from normal subjects. To demonstrate a significant amount of hemolysis of the patient's corpuscles, it was, however, found to be necessary to add acid or carbon dioxide to the serum to compensate for the alkalinity which followed loss of carbon dioxide when the serum was exposed to air. Without addition of acid there was little or no hemolysis. The factor in serum causing hemolysis was found to be thermolabile, for heating to 56°C quickly abolished its hemolytic activity, and characteristically, the hemolytic activity of heat-inactivated serum could not be re-established by the addition of fresh guinea pig serum. In contrast to the behavior of the patient's erythrocytes, normal corpuscles were *not* hemolysed by the patient's serum.

These observations on hemolysis in vitro have been confirmed in vivo, transfusion experiments employing the Ashby method of differential agglutination<sup>6</sup> have demonstrated that normal erythrocytes survive for a normal length of time after transfusion into patients suffering from nocturnal hemoglobinuria.<sup>7, 8</sup> Moreover, transfused normal erythrocytes separated by differential agglutination from those of the recipient after ten days in the recipient's circulation did not undergo hemolysis in vitro when resuspended in acidified serum.<sup>9</sup> On the other hand, it has been recently shown that when patient's corpuscles are transfused to a normal recipient, a proportion is rapidly destroyed.<sup>10</sup> The results of these transfusion experiments are thus essentially similar to those which have been obtained in congenital hemolytic anemia<sup>6</sup> and in sickle cell anemia,<sup>11</sup> disorders in which tests in vitro also indicate that the erythrocytes themselves are abnormal. It was clearly a failure to appreciate the importance of pH that had led the

From the Department of Pathology Postgraduate Medical School of London, London E 8 1 2 3



majority of earlier workers to report that tests for hemolysis *in vitro* were negative in this disease. Van den Bergh,<sup>1\*</sup> however, as far back as 1911, using carbon dioxide as acidifying agent, obtained positive results, and in part he anticipated later observations.

At the present time this unusual corpuscular sensitivity to hemolysis in acidified serum is widely utilized in the diagnosis of nocturnal hemoglobinuria, and has often been referred to as Ham's test. The mechanism behind the effect is, however, still obscure, and its essentially nonspecific nature not generally appreciated. In this paper some further details are presented concerning the effect of pH on hemolysis *in vitro* in nocturnal hemoglobinuria, and this is followed by a consideration of the specificity of the acidified (acid) serum test and its use in diagnosis. Finally, the nature of the hemolytic mechanism in nocturnal hemoglobinuria is discussed. Three patients have been available for study. Earlier observations made upon them have been previously reported.<sup>7, 10, 13, 14</sup>

### HEMOLYSIS *IN VITRO* IN NOCTURNAL HEMOGLOBINURIA

As has already been shown in an earlier publication,<sup>12</sup> the acid-serum test depends upon the adjustment of the pH of the serum to an optimum by the addition of acid. If differing amounts of either hydrochloric, sulphuric or lactic acids are added to equal volumes of serum before the addition of the suspension of corpuscles, a pH-hemolysis curve may be obtained.<sup>13\*</sup> It will then be seen that hemolysis of patient's erythrocytes in serum is maximal at pH 7.0 to 7.4, that is at the physiologic level or a little below, and is inhibited above pH 8 and below pH 6.† As has already been mentioned, serum allowed to stand exposed to the air loses carbon dioxide, with the result that its pH ultimately rises to about 8. This slight alkalinity explains the negative tests for hemolysis which result if the effect of pH is neglected and acidification omitted.

However, hemolysis in unacidified human serum may be observed if a small amount of fresh guinea pig serum is added to it. If the pH range for hemolysis is ascertained after the addition of guinea pig serum, the curve will be found to be skewed, not only is the total amount of hemolysis increased within the range pH 6-8, but hemolysis takes place well to the alkaline side of pH 8 (fig. 1). This is because under these conditions hemolysis in part results from the presence of anti-human heterolysin in the guinea pig serum, to which the patient's corpuscles are unusually sensitive.‡ (see later). Skewness of the pH-hemolysis curve is abolished and hemolysis merely increased within the pH range 6-8, if the anti-human antibodies are removed from the guinea pig serum by previous absorption with normal erythrocytes. This increase in hemolysis is probably due to an increase in serum complement.

\* pH hemolysis curves very similar to those recorded by Dacie and Richardson<sup>13</sup> have by now been obtained using the erythrocytes from 5 patients in all.

† These pH values refer to readings made after the addition of the suspension of corpuscles and after 1 hour's incubation at 37°C.

‡ The use of guinea pig serum in the diagnosis of nocturnal hemoglobinuria is however *not* recommended. The titer of the heterolysin is an unknown factor and normal corpuscles may be hemolyzed to some extent also.

The wide pH-hemolysis range for the hemolysis of patient's corpuscles by human serum and fresh unadsorbed guinea pig serum containing anti-human heterolysin corresponds to the pH range for the action of guinea pig complement,<sup>15</sup> and is similar to the pH range for the hemolysis of human erythrocytes by human complement and isohemolysin<sup>16</sup>, in each case, hemolysis takes place well to the alkaline side of pH 8 and is readily observed without it being necessary to acidify the serum.

### *The Acid-Serum Diagnostic Test*

As already indicated, the acid-serum test depends upon the adjustment of pH to an optimum level for the action of the serum factor (the nature of which is dis-

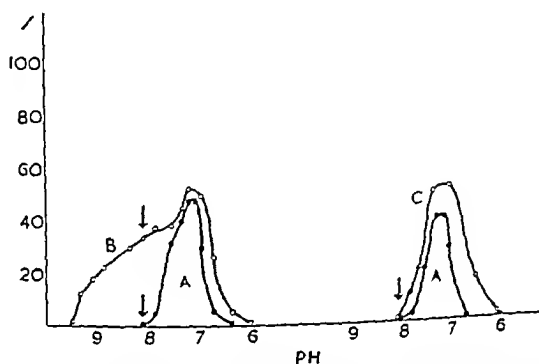


FIG. 1. pH hemolysis curves (A) for the hemolysis of patient's cells in fresh human serum. Curve B represents the curve obtained with the addition of fresh guinea pig serum; the extension to the left is due to the presence of anti-human heterolysin. Curve C shows the effect of addition of guinea pig serum from which all heterolysin had been absorbed; there is an increase in hemolysis but the curve is of the same shape as with human serum alone. The black arrows indicate the amount of hemolysis obtained when serum was used to which no acid or alkali had been added. Fresh guinea pig serum at a final concentration of 1:12 was used in the above experiment. Anti-human heterolysin was absorbed from it by adding to it an equal volume of washed packed human erythrocytes and allowing the mixture to stand for two hours at 4°C before centrifuging. pH was measured with a glass electrode after the suspension had been incubated at 37°C for one hour and hemolysis measured approximately against standards.

cussed in a later section of this paper). For this purpose, Ham<sup>7</sup> recommended the addition to serum of 5 per cent by volume of 0.85 normal lactic acid or N, HCl. However, the amount of acid required for maximal hemolysis needs to be judged carefully, for its effect will vary with the buffering power of the serum proteins and with the method of obtaining serum, and also with the strength of the suspension of corpuscles subsequently added. If serum is obtained by defibrinating blood in an open flask, a procedure which results in the rapid oxygenation of the blood, loss of carbon dioxide and a rise in pH to about 8.0, 10 per cent by volume of N, HCl is then generally the right amount of acid to add for maximal hemolysis. If 10 per cent by volume of a 50 per cent suspension of washed patient's corpuscles is finally added. It should be stressed that in nocturnal hemoglobinuria hemolysis in acidified serum is not the result of increased corpuscular sensitivity to the acid.

of acid per se. That this is so can be shown by suspending patient's corpuscles in serum previously heated to 56 C for 10-30 minutes, under these circumstances, hemolysis within the pH range 6-8 will not take place, and the corpuscles are not clearly distinguishable from normal erythrocytes (fig. 2).

Nevertheless, increased corpuscular sensitivity to acid may be encountered, and this may lead to errors in diagnosis unless this possibility is appreciated. In con-

TABLE 1 — Hemolysis of Erythrocytes from Patient with Idiopathic Acquired Hemolytic Anemia in Acidified Serum

	Tube						
	1	2	3	4	5	6	
	Strength of HCl (per cent)						
	0	$\sqrt{20}$	$\sqrt{10}$	$\sqrt{5}$	$\sqrt{4}$	$\sqrt{3}$	$\sqrt{2.5}$
<i>Patient's serum</i>							
(a) Lysis 10 min at 20 C	0	0	0	8	20	45	60
(b) 2 hr at 37 C	0	0	0	10	20	45	†
<i>Inactivated patient's serum</i>							
(a) Lysis 10 min at 20 C	0	0	0	7	15	45	60
(b) 2 hr at 37 C	0	0	0	7	15	45	†
<i>Normal serum</i>							
(a) Lysis 10 min at 20 C	0	0	0	7	13	35	
(b) 2 hr at 37 C	0	0	0	8	14	40	
<i>Inactivated normal serum</i>							
(a) Lysis 10 min at 20 C	0	0	0	6	12	35	
(b) 2 hr at 37 C	0	0	0	6	12	35	
Approximate pH (after addition of suspension of corpuscles and incubation)	8.0	7.7	7.5	7.0	6.7	6.0	5.3

\* Each tube contained 0.5 ml. serum, 0.05 ml. acid and 0.05 ml. 25% suspension of corpuscles in saline.

† Supernatant brownish due to formation of acid hematin.

genital or acquired hemolytic anemia, the erythrocytes, if sufficiently spherocytic, will undergo hemolysis in normal serum at a pH (6-7) at which normal corpuscles do not hemolyze. In this way, the acid-serum test may appear to be positive. Under these circumstances, however, contrary to the findings in nocturnal hemoglobinuria, hemolysis will not be prevented if the serum is inactivated by heating.

The two experiments described briefly below illustrate the increased sensitivity of spherocytes to hemolysis in acidified sera.

1. The blood was derived from a patient R. L. suffering from idiopathic acquired hemolytic anemia. Anemia was severe, hemoglobin 4.6 Gm., erythrocytes 1,200,000 per cu. mm. with 50 per cent reticulocytes. There was marked spherocytosis and a considerable increase in osmotic fragility. Hemolysis com-

mened at 0.75 per cent NaCl, with 50 per cent hemolysis at 0.54 per cent NaCl and complete hemolysis at 0.30 per cent NaCl. The Coombs test was strongly positive.

Defibrinated patient's blood was centrifuged and the deposited erythrocytes well washed in isotonic saline and finally resuspended as a 5 per cent suspension. Part of the patient's serum was heated to 56°C.

TABLE 2.—*Hemolysis in Acidified Inactivated Serum of Erythrocytes from a Normal Subject from a Patient with Nocturnal Hemoglobinuria and from a Patient with Congenital Hemolytic Anemia*

	Tube*						
	1	2	3	4	5	6	
	Strength of HCl						
	0	1/10	1/5	1/4	1/3.5	1/3	1/2.5
Normal corpuscles, Lysis 30 min at 37°C	0	0	0	0	0	0	90%
Corpuscles from patient with nocturnal hemoglobinuria, Lysis 30 min. at 37°C	0	0	0	0	0	0	70%
Corpuscles from patient with congenital hemolytic anemia, Lysis 30 min at 37°C	0	0	7%	11%	16%	18%	27%
Approximate pH (after addition of corpuscles and incubation)	8.2	7.5	7.2	6.9	6.4	6.1	5.5

\* Each tube contained 0.5 ml. serum, 0.05 ml. acid and 0.05 ml. of a 50% suspension of corpuscles in saline.

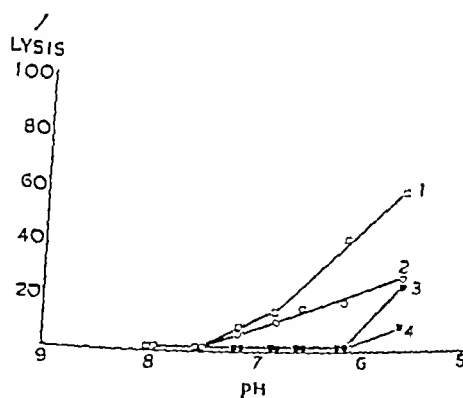


FIG. 2. pH hemolysis curves using human serum heated to 56°C for thirty minutes. The curves for normal corpuscles (1) and for corpuscles from nocturnal hemoglobinuria (2) are not hemolyzed at a pH above 6. The curves for corpuscles from cases of (3) acquired hemolytic anemia and (4) congenital hemolytic anemia are hemolyzed at a higher pH and would give a positive acid serum test. The curves were obtained by adding 10 per cent by volume of hydrochloric acid ranging in strength from 1/20 to 1/2.5 to the serum. The corpuscles were added 0.05 to give a final suspension of 5 per cent.

thirty minutes the remainder was kept frozen in the refrigerator until use. Five ml. of each of the fresh serum and of the heated serum were delivered into two series of small tubes. Serum 0.05 ml. and 0.05 ml. volumes of a range of strengths of hydrochloric acid added to each tube. The tubes had been well mixed into the serum 0.05 ml. volumes of the corpuscles were added to each tube.

10 each tube. The tubes were gently centrifuged after the addition of the suspension of corpuscles had been completed (i.e. after approximately ten minutes at room temperature) and hemolysis estimated by visual comparison with standards made from hemolyzed patient's blood. The deposited corpuscles were then resuspended and the two series of tubes incubated for two hours at 37° C. The tubes were then re-centrifuged and hemolysis estimated as before.

The above procedure was repeated using freshly withdrawn compatible normal serum instead of the patient's serum. The results are given in table 1.

2. The above experiment was repeated using washed corpuscles from a normal subject and from patients with nocturnal hemoglobinuria and congenital hemolytic anemia. This last patient was a young woman aged 27 with moderate anemia, hemoglobin 9.8 Gm. and erythrocytes 3,300,000 per cu. mm. with 13 per cent reticulocytes. There was a considerable increase in osmotic fragility; hemolysis commenced at 0.72 per cent NaCl with 50 per cent hemolysis at 0.47 per cent NaCl and complete hemolysis at 0.30 per cent NaCl. The Coombs test was negative.

Normal serum inactivated by heating to 56° C. for thirty minutes was used and acidified before the addition of the corpuscle suspensions as described for the previous experiment. The tubes were centrifuged after incubation for thirty minutes at 37° C. Hemolysis was estimated by diluting volumes of the supernatants 10 N/10 NaOH and estimating the liberated hemoglobin as alkaline hematin. The pH of the tubes in which there was no hemolysis was measured approximately after incubation and centrifugation by means of the indicators brom thymol blue and methyl red. The results of this experiment are given in table 2 and in figure 2.

The onset of hemolysis appears to be due to the increased sensitivity of spherocytic corpuscles to hemolysis produced by a fall in pH, as well as to the slight alterations in tonicity resulting from the addition of 10 per cent by volume of dilute acid to the serum. Bittorf,<sup>17</sup> in 1914, reported that the resistance to acid of erythrocytes from patients with congenital hemolytic anemia was decreased, but gave no details of his experiments, and little attention seems to have been paid to this phenomenon. The effect of pH on the swelling of erythrocytes in isotonic and hypotonic media has been admirably demonstrated by Hampson and Maizels<sup>8</sup>, in phosphate buffers, increasing acidity causes increasing swelling which reaches a maximum at pH 5.4. Swelling similarly takes place if corpuscles are suspended in serum or plasma of increasing acidity, and it seems likely, therefore, that a major factor in determining the increased liability of spherocytes to hemolyze in acid as well as in hypotonic media is their reduced ability to swell to the same extent as do normal corpuscles.

Of more importance, perhaps, than the appreciation that spherocytes hemolyze unusually easily in acidified sera, is the observation that enhancement of serum hemolytic activity by the adjustment of pH to an optimum by the addition of acid seems not confined to the hemolytic reaction in nocturnal hemoglobinuria. In this laboratory, this effect has been observed in two other quite distinct types of immune hemolytic systems. In each case, as in nocturnal hemoglobinuria while little or no hemolysis occurred in unacidified serum, hemolysis was strikingly obvious if 10 per cent by volume of N/5-N/3 HCl was added to the serum before the addition of the suspension of corpuscles. Thus, a positive acid serum test has been observed in—

(a) *Acute hemolytic (hemolytic) anemia*. In a patient suffering from severe hemolytic anemia, who is the subject of a separate report,<sup>16</sup> an abnormal warm hemolysin was present in the serum, and the patient's corpuscles having absorbed



of guinea pig serum to reactivate inactivated human serum and the sensitivity of patient's erythrocytes to heterohemolysins and isohemolysins (see later) are confirmatory, but need not necessarily be carried out

### *Sensitivity of Patient's Erythrocytes to Other Hemolytic Systems*

There is no characteristic morphologic abnormality of the erythrocytes of patients with nocturnal hemoglobinuria. There may be a considerable degree of macrocytosis, as is often seen in anemia associated with rapid erythrocyte regeneration, but there is no spherocytosis, and osmotic fragility and mechanical fragility are normal. Ham and Dingle<sup>3</sup> have reported normal resistances to saponin and sodium taurocholate, and Shapiro<sup>19</sup> a normal resistance to lysocephalin. Ham and Dingle also made an important observation when they showed that the erythrocytes from a patient with nocturnal hemoglobinuria were more sensitive than normal corpuscles to hemolysis by an anti-human antibody prepared by

TABLE 3—*Diagnosis of Nocturnal Hemoglobinuria: Acidified Serum Test and Necessary Control Observations*

Tube	Fresh human serum	50% saline suspension of corpuscles	Result in nocturnal hemoglobinuria
I	Normal 10 vol	Patient's 1 vol	Trace or no hemolysis
II	Acidified normal* 10 vol	Patient's 1 vol	Considerable hemolysis
III	Inactivated† acidified* normal 10 vol	Patient's 1 vol	No hemolysis
IV	Acidified patient's* 10 vol	Normal 1 vol	No hemolysis

\* Acidified by adding 10% by volume of N/5 HCl

† Inactivated by heating to 56 C for 30 minutes and then acidified by adding 10% by volume of N/5 HCl

immunizing a rabbit with washed human erythrocytes. The patient's erythrocytes were also hemolyzed by human isohemolysin more readily than were normal corpuscles.

These phenomena have been reinvestigated and these increases in sensitivity found to be most striking. Complement is required for hemolysis by isohemolysin but either fresh guinea pig or human serum can be used, and no adjustment of pH is necessary. In the case of one patient of blood group A Rh positive, it was found that the patient's erythrocytes were more sensitive only in respect of hemolysis, when suspended in a range of dilutions of an anti-A serum they were agglutinated to about the same serum dilution as were normal corpuscles. However, when complement was added, in striking contrast to the behavior of the normal corpuscles the patient's erythrocytes were hemolyzed to about the same serum dilution as they were agglutinated. In fact, a proportion of the patient's erythrocytes were

diagnostic test for nocturnal hemoglobinuria. Positive results (obvious hemolysis within six hours of incubation at 37 C) have been observed in acquired hemolytic anemia with marked spherocytosis as well as in nocturnal hemoglobinuria and in hemoglobinuria associated with high titer cold antibodies unless strictest precautions against chilling were taken.

hemolyzed by all of thirteen naturally occurring anti-A sera,\* of agglutinating titers (final dilutions of serum) ranging from 1/8 to 1/1024. That some corpuscles were hemolyzed by a serum with the low agglutinin titer of 1/8 is especially interesting and suggests that even low titer sera have unexpected hemolytic properties. The use of erythrocytes from patients with nocturnal hemoglobinuria

TABLE 4.—*Comparative Sensitivity to Agglutination and Hemolysis by anti A anti D and anti M of Normal Erythrocytes and the Corpuscles from a Patient with Nocturnal Hemoglobinuria*

The patient's corpuscles were agglutinated to approximately the same titer as were the normal erythrocytes, but were much more sensitive to hemolysis. The titers recorded as end points were final dilutions of serum. The corpuscles were washed in saline and used at a final concentration of 1 per cent. Fresh serum from a Group A subject was used at a final dilution of 1/6 as a source of complement. The tubes were kept at 37°C for thirty minutes after the corpuscles had been allowed to sediment at room temperature, end points of hemolysis were read by visual inspection. End points of agglutination were read microscopically after two hours at room temperature in a duplicate series of serum dilutions to which no complement was added. The incomplete anti D serum was titrated in 20 per cent albumin.

Type of serum	Agglutinin titer Patient's corpuscles (Group A)	Hemolysin titer	
		Patient's corpuscles (Group A)	Normal corpuscles (Group A)
1 Anti A	1/8	1/12	0
2	1/16	1/12	0
3	1/32	1/24	0
4	1/64	1/96	0
5	1/64	1/48	1/6
6 (dried)	1/64	1/96	0
7	1/128	1/96	0
8	1/256	1/96	1/3
9	1/256	1/192	1/3
10	1/256	1/384	1/6
11	1/512	1/384	1/3
12	1/1024	1/768	1/6
13	1/1024	1/768	1/3
14 Anti D	1/128	0	0
15	1/128	0	0
16	1/128	0	0
17	1/3000	0	0
18 Anti D (incomplete)	1/1000	0	0
19 Anti M (human)	1/64	0	0
20 Anti M (dried rabbit serum)	1/64	0	0

would thus appear to make more delicate any tests designed to demonstrate the hemolytic properties of anti-A (or anti-B) sera. With Rh antibodies, however, no differences between patient's and normal corpuscles have been demonstrated. The patient's erythrocytes were not hemolyzed by any of four in saline agglutinating antibodies of titer 1/128 to 1/3000 or by an incomplete antibody of titer 1/1000.

I am indebted to Dr. L. Shapiro for technical help with some of these titrations.



in albumin. Similarly, no hemolysis was caused by two anti-M sera of moderate agglutinating titer (table 4).

#### THE SERUM FACTOR REQUIRED FOR HEMOLYSIS AND THE NATURE OF THE HEMOLYTIC MECHANISM

It is not yet clear whether or not the serum factor required for hemolysis is identical with the serum complex known as complement. Evidence suggesting that complement is involved is provided by the fact that guinea pig serum will increase the amount of hemolysis produced by human serum and will, as shown by Ham and Dingle,<sup>3</sup> restore activity to zymine or ammonium hydroxide treated human serum. Moreover, there appears to be a general relationship between a serum's ability to hemolyze patient's erythrocytes at optimum pH and its complement content, as estimated against sheep corpuscles sensitized with rabbit anti-sheep hemolysin, at least in the case of adult human sera. In six experiments utilizing eighteen sera, statistical analysis of the data recorded in table 5 indicates that the two activities probably increase or decrease in parallel ( $p < 0.01$ ). This does not, however, prove that the factors concerned are identical and it might be added that Ham and Dingle<sup>3</sup> could not demonstrate any fixation of complement by patient's erythrocytes in excess of that absorbed by normal corpuscles when acidified serum was subjected to successive absorptions with patient's and normal corpuscles respectively. Unquestionably, however, a specifically human factor is required, for guinea pig serum alone will not cause hemolysis. There is also other evidence which suggests that some factor other than human complement is required, for instance, Ham and Dingle showed that lyophilisation of serum reduced its hemolytic activity without demonstrably altering complement activity against sensitized sheep corpuscles, and that heating to from 45°C to 50°C, filtration through Berkfeld candles and storage at room temperature removed hemolytic activity more readily than complementary activity. Moreover, as has been already referred to, there are marked differences between the pH requirements for hemolysis of patient's erythrocytes in normal serum and the hemolysis by human or guinea pig complement of corpuscles sensitized by isohemolysin or heterohemolysin.<sup>18</sup>

There is thus some evidence for and some evidence against the participation of human serum complement in the hemolysis by serum of the erythrocytes of patients with nocturnal hemoglobinuria, and in the present state of knowledge any theory for the mechanism of hemolysis can only be speculative. It is suggested as a tentative hypothesis that the increased hemolysis *in vivo* is dependent upon a corpuscular abnormality whose effect is to increase the sensitivity of the erythrocytes to certain hemolysins of immune body type. All grades of increased sensitivity are encountered: some cells are extremely easily hemolyzed, others hemolyze less readily and some perhaps behave normally. Presumably, the surface structure of the corpuscles which hemolyze unusually easily is more receptive (there are more receptors) for the hemolysin component of anti-erythrocytic antibodies than in normal corpuscles, which seem to be distinctly insensitive to hemolysis although sensitive to agglutination. This type of abnormality could explain the ease with which patient's erythrocytes are hemolyzed by iso-antibody and hetero-antibody.

Unduly rapid hemolysis *in vivo* could also be explained on these lines if the existence is postulated in all sera of a potentially hemolytic substance of low activity which perhaps fails to affect normal corpuscles seriously, but to which the patient's erythrocytes are fatally sensitive to a greater or less degree because of their hypothetical surface abnormalities. It is admitted that this normally occur-

TABLE 5—*Comparison in Six Experiments of the Hemolytic Activity of Eighteen Fresh Adult Human Sera against Suspensions of Sheep Corpuscles Sensitized with 5 m h d Rabbit anti Sheep Corpuscle Serum and against Nocturnal Hemoglobinuria Erythrocytes*

The serum was used at a final concentration of 1:30 for estimation of its ability to hemolyze the sensitized sheep corpuscles and undiluted and acidified to optimum pH when used with the nocturnal hemoglobinuria erythrocytes. The tubes were incubated in pairs or groups of four for 5 minutes at 37°C and then chilled and centrifuged. The amount of hemolysis in the supernatants was estimated photoelectrically.

The data obtained has been analyzed by the theory of probability. This shows that the correspondence in ranking order of the data in the two columns is most unlikely to have been determined by chance alone ( $p < 0.01$ ).

Experiment	Fresh adult human serum	% hemolysis of sensitized sheep erythrocytes	% hemolysis of nocturnal hemoglobinuria erythrocytes
1	1	14	8
	2	27	11
	3	49	31
	4	56	35
2	5	48	2
	6	57	9
3	7	29	14
	8	52	6
4	9	7	5
	10	44	11
	11	51	10
	12	53	13
5	13	53	3
	14	69	6
6	15	38	1
	16	55	5
	17	71	-
	18	76	17

ing hemolytic substance in serum is only hypothetical and that there is no real evidence of its existence as a separate entity. Nevertheless, for reasons already advanced, it seems almost certain that something different from or in addition to the usual fractions of complement is involved in the hemolysis of patient's corpuscles. If, however, the pH requirements for the absorption of this hemolytic substance are similar to those recently observed<sup>10</sup> as characterizing the par-

hemolysin present in the serum of a patient with acute hemolytic anemia, the effect of pH on hemolysis in vitro would be that which is, in fact, observed in nocturnal hemoglobinuria

### SUMMARY

1 Studies in vitro and transfusion experiments indicate that the cause of nocturnal hemoglobinuria is an abnormality of the erythrocytes. In vitro, patient's corpuscles undergo hemolysis in fresh human sera, but only within a pH range of 6 to 8, a range more restricted on the alkaline side than the limits within which isohemolysis will take place.

2 The nonspecific nature of the acid-serum test is emphasized. In addition to nocturnal hemoglobinuria, positive tests for hemolysis may be obtained in this way with certain warm and cold hemolysins, and in the presence of marked spherocytosis. The control observations necessary for the diagnosis of nocturnal hemoglobinuria are described.

3 The erythrocytes in nocturnal hemoglobinuria are remarkably sensitive to hemolysis by anti-A (or anti-B) but are not hemolyzed by anti-Rh.

4 It is suggested as a hypothesis that the same abnormality, presumably at the corpuscular surface, which is the cause of the increased sensitivity to hemolysis by anti-A results in the erythrocytes being fatally sensitive in vivo to a hemolytic factor distinct from complement and normally present in serum.

### REFERENCES

- <sup>1</sup> HAM T H. Chronic hemolytic anemia with paroxysmal nocturnal hemoglobinuria. Study of mechanism of hemolysis in relation to acid base equilibrium. *New England J Med* 217 915 1937
- <sup>2</sup> ———. Studies on destruction of red blood cells (1) Chronic hemolytic anemia with paroxysmal nocturnal hemoglobinuria. Investigation of the mechanism of hemolysis with observations on five cases. *Arch Int Med* 64 1271 1939
- <sup>3</sup> ———, AND DINGLE J H. Studies on destruction of red blood cells (2) Chronic hemolytic anemia with paroxysmal nocturnal hemoglobinuria. Certain immunological aspects of the hemolytic mechanism with special reference to serum complement. *J Clin Investigation* 18 657 1939
- <sup>4</sup> DACIE J V, ISRAËLS M, C G, AND WILKINSON J F. Paroxysmal nocturnal haemoglobinuria of the Marchiafava type. *Lancet* 1 479 1938
- <sup>5</sup> JORDAN F L F. Etudes sur l'hémoglobinurie. *Acta med scand* 95 319 1938
- <sup>6</sup> DACIE J V, AND MOLLISON P L. Survival of normal erythrocytes after transfusion to patients with familial haemolytic anaemia (acholuric jaundice). *Lancet* 1 550 1943
- <sup>7</sup> ———. Transfusion of saline washed red cells in nocturnal hemoglobinuria (Marchiafava Michel disease). *Clin Sc* 7 65 1948
- <sup>8</sup> MOLLISON P L. The survival of transfused erythrocytes with special reference to cases of acquired hemolytic anemia. *Clin Sc* 6 137 1947
- <sup>9</sup> DACIE J V. Unpublished observations
- <sup>10</sup> ———, AND MOLLISON P L. Survival of transfused erythrocytes from a donor with nocturnal haemoglobinuria. *Lancet* 1 390 1949
- <sup>11</sup> CALLENDER S T E, AND NICKEL, J F. Survival of transfused sickle cells in normal subjects and of normal red blood cells in patients with sickle cell anemia. *J Lab & Clin Med* 32 1397 1947
- <sup>12</sup> VAN DEN BERG, A A H. Ictère hémolytique avec crises hémoglobinuriques. *Rev de méd* 31 63 1911
- <sup>13</sup> DACIE J V, AND RICHARDSON N. The influence of pH on in vitro hemolysis in nocturnal hemoglobinuria. *J Path & Bact* 55 375, 1943

- <sup>14</sup> DACIE J V AND FIRTH A C D Blood transfusion in nocturnal haemoglobinuria Brit M J 1 626 1934
- <sup>15</sup> OSBORN T W B Influence of pH on action of hemolytic complement Biochem J 28 423 1934
- <sup>16</sup> DACIE, J V Hemolysins in acquired hemolytic anemia effect of pH on the activity in vitro of a serum hemolysin Blood 4 928 1949
- <sup>17</sup> BITTORF A Zur pathogenese des chronischen hämolytischen Ikterus Verh deutsch Koogr inn Med 31 619 1914
- <sup>18</sup> HEGOLIN, R, AND MAIER C The heat resistance of erythrocytes A specific test for the recognition of Marchiafava's anemia Am J M Sc 267 624 1944
- <sup>19</sup> SHAPIRO L Personal communication
- <sup>20</sup> HAMPSON, A V AND MAIZELS M The effects of variation in pH on the volume of red cells J Physiol 62 16 (P) 1926

## ACQUIRED HEMOLYTIC ANEMIA

### I THE RELATION OF ERYTHROCYTE ANTIBODY PRODUCTION TO ACTIVITY OF THE DISEASE II THE SIGNIFICANCE OF THROMBOCYTOPENIA AND LEUKOPENIA

By ROBERT S EVANS, M D , and ROSE T DUANE, A B

IT IS NOW evident that the syndrome of acquired hemolytic anemia represents a distinct entity which is separate in pathogenesis and course from the commonly described familial hemolytic jaundice. This distinction, which was recognized by the writers of the early part of the century, was lost sight of by many more recent observers, who suggested that acquired hemolytic anemia was simply a sudden outcropping of a latent inborn defect. Since spherocytosis of the red cells is always present in congenital hemolytic jaundice and is sometimes observed in acquired hemolytic anemia, the confusion was natural, particularly when a sharp distinction could not always be made on clinical grounds. Beginning with the red cell survival experiments of Dacie and Mollison,<sup>1</sup> it has become increasingly evident that acquired hemolytic anemia is caused by a hemolysin \* active for all erythrocytes, while congenital hemolytic jaundice is due to a defect in red cell structure.<sup>2</sup> During the last few years it has been possible to demonstrate sensitization of erythrocytes from patients with acquired hemolytic anemia with immunologic technics developed in the field of Rh sensitization.<sup>3, 7</sup> We have some evidence, then, by analogy, that the hemolytic agent in acquired hemolytic anemia is an immune body similar to the univalent or hyperimmune Rh antibody and may be a response to antigenic stimulus. The ready demonstration of the abnormal immune mechanism in acquired hemolytic anemia elevates this rather rare disease from the position of an obscure hematologic phenomenon of uncertain etiology to the general field of abnormal immunology. Because of the unique properties of erythrocytes, the affected tissue can be isolated and subjected to close observations so that variations in the rate of production of the hemolysin can be measured in relation to severity of the disease and to any type of therapeutic procedure.

It is worthwhile at this point to summarize our knowledge of the antibody like agent which appears to be responsible for the destruction of red cells in acquired hemolytic anemia.

1. The destructive agent appears to be a fraction of plasma protein, probably a globulin, since erythrocytes from persons with acquired hemolytic anemia are agglutinated with the anti-human serum rabbit serum of Coombs, Mourant and Race,<sup>3</sup> as are cells sensitized by Rh hyperimmune antibody. Red cells from normal individuals and from patients with other types of disease are not agglutinated by this reagent.

From the Department of Medicine Stanford University School of Medicine San Francisco Calif

\* The word hemolysin is used as an all inclusive term for agents known to bring about destruction of red blood cells

2 The hemolytic agent is also similar to the univalent or hyperimmune Rh antibody in that sensitized cells do not usually agglutinate in saline but require a more complex colloidal medium. Whole serum, 30 per cent beef albumin and 2 per cent acacia in 1.5 per cent calcium chloride have been found to provide the necessary factors to allow agglutination to take place.

3 Since accelerated hemolysis proceeds at a fairly constant rate in acquired hemolytic anemia, it appears that the agent does not require special conditions of temperature or pH for activity as is the case in some hemolytic syndromes. In keeping with this property of constant activity of the hemolysin it has been our experience that the immune body could be found on the surface of the cell when it could not be demonstrated in the serum.

4 We have been able to remove the sensitizing agent from the surface of the red cell by heating a suspension of the sensitized cells to 56°C. in normal saline.<sup>5</sup> The immune body appeared to remain active to some degree in the saline eluate since normal cells exposed to it became agglutinable in the Coombs reagent. This observation is considered further evidence that the hemolytic agent is an immune body. So far this observation that the lytic agent can be transferred to normal cells has not been confirmed by others, and we have been successful in further attempts in only one of three patients.

5 The agent is active for all erythrocytes since transfused cells appear to be destroyed at a rate which approximates the rate of destruction of the native cells.

Although the etiologic significance of the antibody-like abnormality found in acquired hemolytic anemia appears to be established, the study of patients who appeared to have recovered completely following splenectomy showed a persistence of the abnormality. The erythrocytes from patients in remission were found to be agglutinable in the anti-human globulin serum even though all evidence of accelerated hemolysis had subsided. This observation appeared to throw some doubt on the significance of sensitization as a primary cause of the disease. It seemed possible that the agglutinability of the erythrocytes in the various media could be the result rather than the cause of the disease, and that splenectomy produced a remission by removal of the principal site of destruction of abnormal cells. On the other hand, it appeared more likely that a quantitative relationship between the amount of immune body present and the rate of blood destruction might explain the apparent paradox. We attempted, therefore, to devise a method of quantitating the amount of antibody on the red cell so that measurements could be made during active and quiescent phases of the disease. In this report we are presenting evidence of a direct relationship between the amount of antibody on the cell and the activity of the hemolytic process.

Ten rabbits were injected subcutaneously at weekly intervals with 0.5 cc. of fresh human serum for six weeks. Following an interval of one month a second course of injections was given before the animals were sacrificed and the sera collected. The red cell agglutinins in the rabbit serum were adsorbed by mixing the sera in 5 cc. amounts with equal quantities of a 50 per cent suspension in normal saline of pooled Group A, B and O cells that had been washed repeatedly to remove serum protein. The mixture was then incubated at 37 C. for one hour with frequent agitation before centrifugation and removal of the supernatant fluid. Two such adsorption procedures sufficed to remove all of the cell agglutinins. It was found important to use O cells as well as those of Group A and B, since agglutinins specific for O cells appeared in low titer if O cells were not included in the adsorption process.

Once the rabbit serum was completely adsorbed, it did not agglutinate washed human cells of any group or type in any concentration. It was then filtered, placed in small vials and kept in the frozen state where it maintained its original activity.

The amount of antibody for human serum protein in the adsorbed rabbit serum may be determined by precipitation titers. However, the authors found that the most practical method of assaying the rabbit serum was to determine the highest dilution at which cells sensitized in a uniform manner with Rh<sub>0</sub>

blocking antibody were agglutinated. This was done by exposing Rh<sub>1</sub> cells of one individual to a high concentration of Rh<sub>0</sub> blocking antibodies for one half hour at 37 C. The Rh<sub>0</sub> blocking serum used showed an agglutinin titer of 1-5000 in beef albumin and was used in a dilution of 1-20 to sensitize the Rh<sub>1</sub> cells. The sensitized cells were washed three times in normal saline and added to the saline dilutions of the rabbit serum and incubated for one half hour at 37 degrees before brief centrifugation and microscopic observation of the end point of agglutination. The two sera used have shown a consistent ability to agglutinate Rh<sub>1</sub> cells so sensitized in dilutions of 1-320 plus or minus one dilution.

Red cells from patients with acquired hemolytic anemia tested for sensitization were collected from venous or capillary blood and diluted directly in normal saline. The cells were washed three times with normal saline and a final 2 per cent suspension was approximated. A drop of the suspension was then added to a drop of rabbit serum and left for one half hour at room temperature and subjected to brief centrifugation before observation of agglutination. Cells from patients with active acquired hemolytic anemia showed a nearly complete agglutination in a 1 to 10 dilution of the anti globulin rabbit serum indicating that most if not all the cells were sensitized. Suitable control suspensions showed no agglutination. It was noted that cells from patients with acquired hemolytic anemia were agglutinated by varying dilutions of the rabbit serum, so observations were made to determine if the amount of antibody on the cell surface had an inverse relationship to the concentration of rabbit serum required to produce agglutination. That such an inverse relationship exists is shown by the analogy with cells sensitized by varying concentrations of Rh<sub>0</sub> blocking antibody. Rh<sub>1</sub> cells were incubated one hour at 37 C. in serial dilutions of an anti Rh<sub>0</sub> blocking serum, washed and exposed to dilutions of antiglobulin serum and the end point of agglutination observed microscopically. The results are shown in table 1.

It is evident from the above that cells exposed to decreasing amounts of sensitizing antibody, below a concentration which saturates (1-20 to 1-320) require increasingly high concentrations of antiglobulin serum to produce agglutination. From this it may be inferred that avidity of patient's cells to agglutinate in the dilutions of the rabbit sera is proportional to the amount of antibody on the cell surface.

In the observations reported here we have used two preparations of antihuman globulin serum. Both show comparable activity for agglutination of Rh<sub>0</sub> cells that have been sensitized by blocking antibody. However, we have observed certain variations in the ability of these sera to agglutinate cells from patients with active disease. The cells of some patients show a consistently greater susceptibility to agglutination in one serum than the other. These observations indicate the importance of using several rabbit sera simultaneously to detect sensitization of erythrocytes in acquired hemolytic anemia.

#### DESCRIPTION OF PATIENTS

Eleven patients with acquired hemolytic anemia are included in this series. One of the patients has been described in detail in a previous report.<sup>8</sup> The diagnosis of hemolytic anemia in each patient was based on the presence of a chronic anemia,

reticulocytosis, an increase in serum bilirubin and, in most patients, the demonstration of an increased fecal urobilinogen. All patients showed erythroid hyperplasia of the bone marrow. The patients in the series were grouped together under the heading of acquired hemolytic anemia because of an absence of a family history of anemia or jaundice and a lack of any personal history suggestive of hemolytic anemia prior to the onset of the present illness in adult life. In most instances, patients did not exhibit well-marked spherocytosis and increased osmotic fragility.

TABLE 1.—*Relation of Amount of Antibody on the Cell Surface to Agglutinability of Sensitized Cells in Dilutions of Anti globulin Serum*

Dilutions of anti globulin serum	Dilutions of anti Rh serum						
	1-20	1-320	1-640	1-1200	1-2400	1-5000	1-10 000
1-5	++++	++++	+++	+++	++	++	+
1-10	++++	++++	+++	+++	++	+	0
1-20	++++	++++	+++	++	++	0	0
1-40	++++	++++	++	++	+	0	0
1-80	+++	+++	++	+	0	0	0
1-160	++	++	+	0	0	0	0
1-320	+	+	0	0	0	0	0
1-640	0	0	0	0	0	0	0

TABLE 2.—*Basic Data of 11 Patients with Acquired Hemolytic Anemia*

Pt. Sex Age	Known factor associated with onset	Hematocrit	Hemoglobin Gm. 100 cc	RBC Million per cmm	Reticulocytes %	Icterus Index	Fecal urobilinogen Mg. day
C. S. F. 22	0	21	7.7	2.2	8	30	2060
C. A. F. 24	Pneumonitis	18.5	6.0	1.6	9	50	
W. G. M., 31	Hepatitis & Sulfonamides	26	9.5	2.46	18	20	450+
J. F. M., 34	0		4.1	1.1	20	15	
H. S. F. 37	Gold therapy for arthritis	27	9.8	2.75	13	40	950
F. R. F. 42	Pregnancy thrombopenic purpura	28	9.5	- 5	—	10	530
O. W. F. 47	0	23	7.5	- 5	1-	10	1360
A. D. M., 50	Injury	34	10.2	3.3	13	20	142
D. B. F. 55	0		5.3	1.6	9	10	4-5-
B. T. F. 64	0	28	9-	- 9	8.5	15	152
E. S., M., 78	Chronic lymphatic leukemia	26	7.8	- 3	20	30	

In further distinction to congenital hemolytic jaundice, the rapid destruction of normal transfused cells was evident when measured in seven of the patients. Other varieties of hemolytic anemia were excluded by appropriate tests.

In 5 of the 11 patients there was nothing to suggest a precipitating cause for the onset of hemolysis. In 6 of the patients there were a variety of conditions associated with onset of the disease which have been recorded in table 2, along with a summary of the basic data during the first few days of observation. The data



varied in severity from the mild to the very acute form, and in all patients the course was prolonged over a period of weeks, months and even years, so there was ample opportunity to make serial observations to confirm the initial data recorded in the table

## RESULTS

### *1 Activity of the Hemolytic Process in Relation to the Amount of Antibody on the Cell Surface as Measured by Agglutinability of the Erythrocytes in the Anti-globulin Serum*

For this purpose the patients may be divided into three groups as follows

1 Four patients were studied with the Coombs reagent in both the active phase and during a remission. In 2 of the cases (Nos. 1 and 7) the remission followed a splenectomy, and in the other 2 (Nos. 3 and 8) the remission occurred spontaneously after weeks of observation of the active state.

2 Two patients with persistently active disease did not have splenectomy. One of these (No. 2) died without benefit of splenectomy after several weeks of observation. The second patient is an elderly man (No. 11) who has hemolytic anemia in association with chronic lymphatic leukemia. Results of x-ray therapy will be discussed below.

3 Five patients have been studied with the antiglobulin serum technic following splenectomy. Two of these patients (Nos. 4 and 5) have active disease with anemia and rapid blood destruction, although there was evidence of some improvement following splenectomy. Three patients (Nos. 6, 9 and 10) were studied at periods of one year to eighteen months after continued remissions induced by splenectomy. In all examinations the erythrocytes showed agglutination in the antiglobulin serum.

In general, good correlation was found between activity of the disease and the amount of antibody on the surface of the red cell as measured by the technic described above. As shown in table 3, erythrocytes of patients with active disease were agglutinated by dilutions of antiglobulin serum which ranged from 1-80 to 1-1280. On the other hand, the erythrocytes of patients in whom the disease process had subsided so that the rate of hemolysis approached normal were agglutinated in ranges of 1 to 2 dilution up to 1-80.

There seemed to be some variation between individual patients as to the amount of antibody on the surface of the red cells in the active phase of the disease as compared to the amount present during a complete or partial remission. While most patients with active disease showed agglutination of cells by 1-160 to 1-320 dilution of the anti-globulin serum during the active phase of the disease, one patient (E. S., No. 11) had erythrocytes which were frequently agglutinated by dilutions of 1-1280 or 1-2500. On the other hand, the red cells of A. D., No. 8, who had a somewhat milder degree of anemia, were never agglutinated by dilutions greater than 1 to 80. However, when this patient entered a remission, which for a time appeared nearly complete, agglutination of erythrocytes was not present in dilutions greater than 1-5.

An exception to the generalization concerning agglutinability of the cells and

activity of the disease was observed in a patient (H. S., No. 5) in whom the disease seemed to be persistently active but whose erythrocytes on several occasions showed diminished susceptibility to agglutination as shown in table 4. There seemed to be no relationship between the severity of the anemia and the reticulocytosis to the amount of immune body on the surface of the red cell during the periods of observation. There are several possible explanations of this observa-

TABLE 3.—*Typical Hematologic Data in Relationship to Agglutinability of the Erythrocytes in Dilutions of the Anti globulin Serum*

Patient	State of Disease	Hematocrit	Reticulocytes	Icterus Index	Fecal Urobilinogen	Greatest dilution of anti globulin serum showing agglutination
1. C. S.	Active	21	8.0	30	2080	1-3-0
	Quiescent post splenectomy	41	1.2	5	125	1-20
2. C. A.	Active	28	22.0	100		1-64
3. W. G.	Active	26	18.0	20	450*	1-160
	Spontaneous remission	36	12.0	10		1-10
4. J. F.	Active disease after splenectomy	17	70.0	40		1-160
5. H. S.	Active disease after splenectomy	26	20.0	30	700	1-3-0
6. F. R.	Quiescent after splenectomy	40	1.0	10		1-40
7. O. W.	Active	23	12.0	10	1360	1-3-0
	Quiescent 3 months after splenectomy	33	1.6	8	20	1-1
8. A. D.	Active	34	13	20	1800	1-64
	Spontaneous remission	45	1%	10		1-5
9. D. B.	Quiescent 18 months post splenectomy	42	0.5			1-5
10. B. T.	Quiescent 12 months post splenectomy	42	4.0		3-6	1-5
11. E. S.	Active	28	--	30		1-6

The method is rough at best, and perhaps variations in technique of preparation of the cells accounted for loss of some immune body in those observations when the cells were not agglutinated by higher dilutions of the rabbit serum. Also, there may be fairly rapid variations in the amount of antibody present which are not reflected in the degree of anemia or reticulocytosis.

A second exception to the generalization that activity of the disease is related closely to the amount of antibody on the cell was observed in W. G. who entered a phase of spontaneous remission after six weeks of active disease with a well marked drop in agglutinability of the erythrocytes and then showed a return

the susceptibility of his cells to agglutination in higher dilutions (1-160) of the rabbit serum without immediate return of active anemia as shown in table 5

Of particular interest are the two patients (Nos 1 and 7) who were studied before and after splenectomy, since they provide data as to the mechanism of the response to splenectomy. In both patients there was a decrease in the agglutinability of the red cells in antiglobulin serum and a cessation of abnormal hemolysis during the week following splenectomy as shown in table 6

TABLE 4—Serial Observations on a Patient with Active Hemolytic Anemia whose Cells Occasionally Seemed to Show Diminished Amount of Antibody without Varying the Activity of the Distast

Date	Hematocrit	% Reticulocytes	Greatest dilution of antiglobulin serum showing agglutination
9/22/47	24	22	1-320
9/29/47	27	20	1-10
10/13/47	25	20	1-160
10/21/47	24	39	1-320
11/13/47	23	24	1-40
1/13/48	28	20	1-40
1/29/48	23		1-20
3/ 8/48	24	22	1-320
3/22/48	22	21	1-320
4/ 6/48	24	21	1-160

TABLE 5—Spontaneous Remission Associated with or Following Diminished Agglutinability of the Red Cells. So far there has been no evidence of recurrence after the cells again became susceptible to agglutination in dilutions of 1-160

	Hematocrit	Reticulocytes	Antiglobulin serum dilution
7-17	27	9.5	1-320
7-28	31	7.7	1-160
8-2	32	11.5	1-160
8-13	32	12.5	1-10
8-20	36	13	1-1
9-10	36	12	1-80
9-27	45	3.0	1-160
10-30	49	0.9	1-160

One of the patients (C S) showed a fall in the hematocrit and a rise in icterus index to 15 six weeks after splenectomy, accompanied by evidence of an increase in the amount of erythrocyte antibody on the cells, but no further evidence of relapse occurred. She was well and free of signs and symptoms of hemolytic disease six months later. The second patient (O W) had a relapse and died in a distant part of the state about three months after our last observations showed the process to be quiescent.

Serial observations have also been made of one patient (E S) during the course of x-ray therapy of chronic lymphatic leukemia. The essential data are shown in table 7.

TABLE 6—Data Showing the Effect of Splenectomy in Two Patients. There was rapid hematologic improvement and a drop in the concentration of antibody on the cell surface following splenectomy.

Patient	Hematocrit	Reticulocytes	Icterus index	Fecal Urobilinogen Mg./day	Greatest dilution of the anti-globulin serum producing agglutination
1. C. S., F. 44					
Before splenectomy	19	7.0	20	2080	1-320
1 wk. after splenectomy	43	1.0	5	210	1-1
6 wk. after splenectomy	35	1.6	15	125	1-160
6 mo. after splenectomy	41	1.1	5		1-20
2. O. W. F. 47					
Before splenectomy	23	12.0	10	1360	1-320
1 wk. after splenectomy	36.5	1.5	8		1-10
3 mo. after splenectomy	33	1.6	8	220	1-1

TABLE 7—Serial Observations in Patient with Active Acquired Hemolytic Anemia and Chronic Lymphatic Leukemia in Relation to X-ray Therapy. There appeared to be some lessening of the hemolytic process, improvement in the anemia and possible reduction in the amount of antibody on the cell following the second course of x-ray therapy which depressed the lymphocyte count in the peripheral blood.

	Hematocrit	Reticulocytes	Leukocytes per cu. mm.	X-ray	Highest dilution of anti-globulin serum producing agglutination
		%			
1-22	28	22	90,000	80 r total body radiation	1-640
1-29	28	15	16,000		1-160
2-11	28	15	24,000		1-320
2-20	26	17	24,000		1-320
3-15	27	17	22,000		1-640
4-12	23	18	28,000	600 r Total to Spleen 5-13-10 6-1-10 6 doses	1-1280
5-3	20	29	37,500		1-2500
5-13	23	19	40,400		1-640
5-17	16	26	36,000		1-160
5-20	16		42,000		1-320
5-24	13	58	22,500		1-640
5-27	14	41.8	17,640		1-1280
6-1	21	21	5,000		1-320
6-4	17	19	5,400		1-2500
6-7	16.5	32	7,000		1-1280
7-2	27	7.6	5,000		1-160
8-20	32		2,400		1-80
9-8	30	8.2	8,500		1-320
9-15	30.5	10.0	7,500		1-160

It is of interest that there was an improvement in the anemia following a reduction in the lymphocyte count in the response to x-ray therapy. At the same time the amount of antibody on the cells appeared to reach lowest concentration of any time during his course. These variations may be chance variations in the disease and bear no relationship to x-ray therapy. However, exposure to x-ray has been shown to diminish antibody response in animals, and we have previously observed improvement in a patient with acquired hemolytic anemia<sup>9</sup> given x-ray therapy. With a method of assay available, further data may be obtained on the effect of x-ray on the amount of antibody produced in acquired hemolytic anemia.

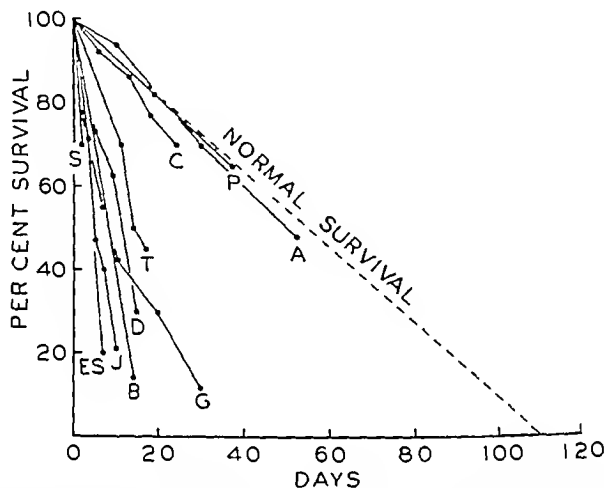


FIG. 1.—Survival time of transfused cells in patients with acquired hemolytic anemia and in patients with other types of hemolytic disease. Patients S, E, S, J, B, T, G all showed sensitization of the erythrocytes. Patients C, P, and A had congenital hemolytic jaundice, Mediterranean anemia, and paroxysmal nocturnal hemoglobinuria.

## II The Longevity of Transfused Cells

Observations of the survival of normal transfused cells was made by the technique of differential agglutination in 6 of the 11 patients. The results of these observations are shown in figure 1. In all 6 cases the destruction of the transfused cells was several times the rate of destruction of transfused cells in normal individuals and in patients with other types of hemolytic disease. Observations in figure 1 include congenital hemolytic jaundice, Mediterranean anemia, and paroxysmal nocturnal hemoglobinuria.

In three additional patients in this series (Nos. 2, 4, 5), repeated transfusions were necessary, and it soon became apparent from simple calculation that the transfused red cells were being destroyed rapidly because of the transitory effect on the severity of the anemia. In each case enough red cells were given during the space of a few days to replace entirely the patient's cells and to produce a

normal or greater than normal circulating red cell volume. Since the anemia quickly developed again in the absence of blood loss, it must be assumed that the transfused, as well as the patient's own red cells were rapidly destroyed. In one of these patients (No. 5) exhibiting very marked spherocytosis, it was shown previously<sup>4</sup> that transfused cells showed a tendency toward spherocytosis and an increase in hypotonic fragility within forty-eight hours after injection.

In the remaining 2 patients, no data were obtained as to the longevity of transfused cells.

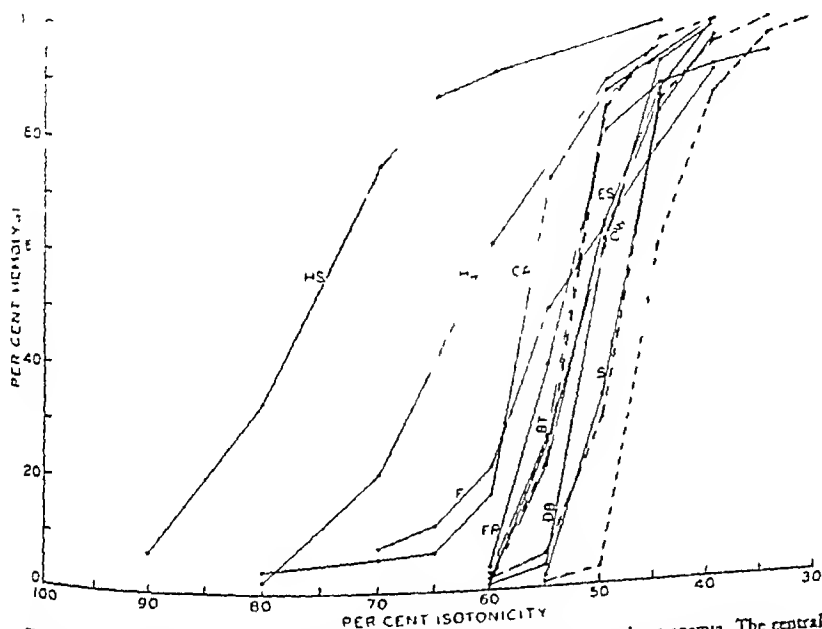


FIG. 2.—Curves of hypotonic fragility in 12 patients with acquired hemolytic anemia. The central dotted line is the average of 30 control determinations while the lateral lines represent the extremes. H S, H J and J F were patients who continued to have active disease after splenectomy. There is evidence that the curve of hypotonic fragility was close to normal prior to splenectomy. C A showed the most severe disease prior to splenectomy. One H S showed a normal fragility curve even though the disease was very active.

### III Susceptibility to Hemolysis in Hypotonic Solution

Representative samples of the quantitative curves of hypotonic fragility are shown in figure 2. An average control curve is shown and the limits of variation of some 30 control curves are also represented. It can be seen that most of our patients with acquired hemolytic anemia had curves of hypotonic fragility which were increased above average but were close to or within the widest limits of normal variation by the method employed. In one patient (C A) with active hemolytic anemia and an output of 2080 mg. of fecal urobilinogen per day, the curve of hypotonic fragility was identical with a normal curve done at the same time.

On the other hand, 3 patients in this series (H S, J F, C A) and one other patient (H J), reported elsewhere,<sup>9</sup> showed curves of hypotonic fragility which were considerably increased above the normal range. Three of these 4 continued to have chronic hemolytic anemia following splenectomy. The fourth patient (C A) showing greatly increased hypotonic fragility of the red cells had by some criteria the most severe hemolytic anemia in the series and died without splenectomy.

Sweeping conclusions cannot be drawn from these observations, but it is evident that the susceptibility of circulating erythrocytes to hemolysis in hypotonic solution may be quite normal in the presence of active disease even with sensitive methods of measurement. The increase in hypotonic fragility when the disease persists following splenectomy suggests that the spleen *in situ* may remove the spherocytic cells from the circulation and keep the curve of hypotonic fragility in

TABLE 8 — *Platelet and Leukocyte Counts before and after Splenectomy in Patient who Showed Thrombocytopenia or Leukopenia along with Active Hemolytic Anemia*

Patient	Platelets		Leukocytes	
	Before splenectomy	Following splenectomy	Before splenectomy	Following splenectomy
C S	70,000	100,000	2,000	7,000
J F	60,000	70,000	13,000	18,000
F R	10,000	45,000	9,500	6,700
O W	12,000	675,000	1,700	8,000
D B		190,000		Normal
	35,000 106,000	Normal	5,000	

the peripheral blood close to normal range unless the disease becomes very active, as was the case in the patient (C A) who died without splenectomy.

#### IV *Thrombocytopenia and Leukopenia*

Five patients in this series had persistently low platelet counts prior to splenectomy, and 2 of the 5 had a marked degree of leukopenia. These findings are summarized in table 8, which shows representative platelet and leukocyte counts before and after splenectomy.

Only one of the patients with thrombocytopenia had clinical manifestations of purpura. This patient (F R) was hospitalized because of purpura, and the hemolytic anemia was not suspected at first. The remaining 4 patients did not exhibit purpuric manifestations, although the platelet count was below 60,000 per cu mm in several determinations. Following splenectomy, the platelet counts of three of the five patients rose to a normal level or above, concomitant with a subsidence of the hemolytic process. The patient with thrombocytopenic purpura and hemolytic anemia continued to have few platelets in the peripheral blood with platelet counts of 10,000 or less for the remaining two months of her pregnancy and during the postpartum period. In spite of the failure of her platelets to rise, there appeared to be a definite improvement in the purpura after operation, and

the bleeding time fell to a normal range. The hemolytic anemia improved slowly, beginning about one month following splenectomy, but was still active at the time of delivery. She was delivered without incident with one transfusion given at the time of delivery. The platelet count one year later was still only 50,000 per cu mm, and there was evidence of some sensitization of the erythrocytes, though the hematocrit was 40 and the reticulocytes were 0.5 per cent.

A second patient (J. F.) continued to exhibit a persistent thrombocytopenia following splenectomy. The chronic hemolytic anemia also persisted without real remission, although there was evidence of some decrease in severity following operation.

Both patients with leukopenia showed a prompt and consistent elevation of the leukocyte count to normal range following splenectomy, along with a response of the other blood elements.

### DISCUSSION

The demonstration of the direct relationship of the amount of adsorbed antibody to the rate of destruction of red cells is another step in our understanding of the pathogenesis of acquired hemolytic anemia. Activity of the disease is associated with evidence of maximal adsorption of the immune body, whereas remission in the hemolytic activity is, in the main, associated with distinctly less adsorbed immune substance. So far we have not observed the complete disappearance of the abnormality, even in patients who have been in remission for a year or more, which explains perhaps why relapse of this disease occurs so readily. The immediate effect of splenectomy when it is successful in producing a remission appears to be brought about by a sharp reduction of the amount of adsorbed sensitizing agent on the cell. This suggests that the spleen is the principal site of production of the sensitizing agent. Wagley and co-workers<sup>10</sup> have recently been able to demonstrate the persistence of the sensitizing agent in the washed pulp of spleens from patients with acquired hemolytic anemia. The failure of splenectomy to produce a remission in some patients is evidently due to the production of sufficient hemolysin in other lymphatic or reticulo-endothelial tissues to keep the disease active. Even when the hemolytic process continues there is usually evidence that splenectomy has diminished its severity. This observation implies that some proportion of the total quantity of hemolysin is always produced in the spleen.

The exact nature of the hemolysin is still not entirely clear. The chief question seems to be whether or not it is a true immune body or some entirely different as yet unknown, type of variant of normal plasma protein. Evidence is needed to show that the hemolytic agent is a specific immune response to an antigen common to erythrocytes. Perhaps a complex of some component of the red cell and a foreign substance such as a virus or medication provides the necessary antigenic stimulus. This explanation would be more clear-cut if it were shown that hemolytic anemia is definitely associated with sulfonamide medication exhibited the same evidence of sensitization of the erythrocytes. In our series one patient received gold therapy prior to and during the onset of her disease but this cannot be regarded as a



than suggestive evidence that erythrocyte antibody production may be stimulated by a medication, since hemolytic disease is not generally reported as a complication of gold therapy.

Another group of anemias which require further study with the special immunologic technic are the so-called symptomatic hemolytic anemias. This term is used to describe hemolytic disease associated with a large variety of disease states including lymphomas, leukemias and cirrhosis. So far we have studied only one such patient, but the mechanism of hemolysis seems to be the same as in other patients with acquired disease. It is of interest that diseases of lymphocytic tissue, which is known to be active in the production of antibodies, are associated with hemolytic anemia and that treatment of the underlying disease by x-ray is said to be helpful in controlling the hemolytic process.<sup>11</sup> It is probably significant that an improvement in the anemia and transient decrease in the amount of adsorbed antibody followed x-ray therapy of the lymphatic leukemia in our patient.

In the study of patients with atypical or acquired hemolytic anemia several technics should be employed to determine the presence or absence of adsorbed antibody. Our experience, with two separately prepared anti-human serum rabbit sera, indicates that specificity may vary and that two or more sera should be used to demonstrate the presence of adsorbed antibody. Less specific but equally sensitive methods should also be used in conjunction with the Coombs test. Washed erythrocytes should be suspended in human serum and in 30 per cent beef albumin, incubated, subjected to centrifugation and inspected for agglutination before the possibility of sensitization is discarded. The same technics should be employed in an attempt to demonstrate free antibody in the serum. Normal cells will adsorb the free immune body if present in the patient's serum and become agglutinable in the Coombs reagent or in beef albumin. However, our efforts to demonstrate free antibody in the patient's serum have been inconstant as opposed to the consistency with which it has been demonstrated to be adsorbed on circulating cells.

The exact mechanism of cell destruction brought about by the antibody is not entirely clear. We have, as yet, no evidence that hemolysis occurs as a result of lysis with the fixation of complement. There is, on the other hand, evidence to show that destruction of sensitized cells is relatively slow. Observations with antiglobulin serum indicate that the great majority of cells in the peripheral blood are sensitized, since nearly all are involved in the agglutination. However, studies of pigment excretion and observations of longevity of transfused cells indicate a survival time of several days for the average cell. It is evident that sensitization does not bring about immediate destruction.

There is evidence from transfusion experiments to show that sensitization is reversible, since cells from patients with acquired hemolytic anemia may have a normal survival time when transfused into a normal individual.<sup>1</sup> It has also been demonstrated that A cells sensitized by B agglutinin are not irreversibly damaged and exhibit a normal survival time after being used in a transfusion.<sup>12</sup> We have observed that transfused cells which have been in the patient's circulation for several days prior to splenectomy and demonstrated to be involved in the hemolytic process show a normal rate of disappearance when splenectomy has produced a

remission. We may conclude that sensitization brings about cell destruction slowly over the course of days and that it does not immediately damage the red cell irreversibly.

If the sensitized cells are susceptible to agglutination *in vivo* we have an explanation of cell destruction, since it has been shown that the injection of a simple agglutinin, such as Concanavalin A, will produce a hemolytic anemia in animals.<sup>12</sup> We have not observed agglutination of red cells from patients with acquired hemolytic anemia *in vivo*, but agglutination does occur under optimum conditions *in vitro*. When the washed cells are suspended in whole human serum and subjected to light centrifugation, agglutination is usually observed. The reaction is qualitative, but we have the impression that the intensity of the agglutination is proportional to the degree of sensitization as measured by the anti-human-serum serum technic as described above. In several instances, cells from patients in mild or inactive phases of the disease failed to agglutinate when centrifuged in whole serum, probably because of a lack of sufficient concentration of antibody on the cell surface.

It seems probable that agglutination of sensitized cells *in vivo* is the most important mechanism in cell destruction. Agglutination produces stasis of cells which leads to increased osmotic and mechanical fragility and probably susceptibility to phagocytosis. If a critical concentration of immune globulin on the cell surface is necessary to produce agglutination *in vivo* we have an explanation for the cessation of hemolysis in the quiescent state since the amount of antibody on the cell appears to be considerably reduced. The suggestion that a certain concentration of immune body on the cell surface is necessary for cell destruction *in vivo* may explain the absence of hemolytic disease in the newborn even when maternal sensitization has occurred and there is maternal-fetal incompatibility. In these instances it is possible that the concentration of immune substance on the baby's cells may not be great enough to produce agglutination and hemolysis.

The implications of the association of thrombocytopenia and leukopenia with acquired hemolytic anemia are clear. It strongly suggests that the leukopenia and thrombopenia in these patients is due to the presence of an immune body with a broader range of activity than the red cells or to a separate immune substance or substances more specific for platelet and white cell tissue. The latter explanation is more likely since there is no correlation between severity of the hemolytic process and the degree of thrombocytopenia or leukopenia. It is possible that a similar mechanism will be found for thrombocytopenic purpura and splenic neutropenia which occur as single disease states unaccompanied by hemolytic anemia. Previous observations of the occurrence of leukopenia, thrombocytopenia and hemolytic anemia in the same patient were made by Wiseman and Doan<sup>14</sup> in their original report of primary splenic neutropenia, and by Dameshek and Estren.<sup>15</sup> The latter authors refer to these cases as "hypersplenic hemolytic anemia" and a correlation of the leukopenia and thrombocytopenia on the basis of an unusual degree of inhibition upon the bone marrow. Such cases often show a remarkable response to splenectomy. It is probable that the hemolytic anemia in the type of acquired variety, especially since a family history of hemolytic disease is not

ing A patient with splenic neutropenia described by Rogers and Hall<sup>15</sup> showed thrombocytopenia and a mild anemia with slight polychromatophilia, normoblasts in the peripheral blood and elevation of the indirect reacting serum bilirubin Fisher<sup>16</sup> recently commented on the presence of leukopenia and thrombocytopenia in one of a series of patients with acquired hemolytic anemia

The association of thrombocytopenia with acquired hemolytic anemia seems to be more common than the occurrence of a panhematopenia In 1941 we observed severe thrombocytopenia with fatal termination in a 31 year old man who had had splenectomy three years previously for hemolytic anemia that was evidently of the acquired variety since there was no past or family history of hemolytic disorder A mild thrombocytopenia was present with the severe anemia prior to surgery There was response of both anemia and thrombopenia following operation, and he was well until the development of purpura four years later in which blood platelets were close to the zero level He died of a cerebral hemorrhage and autopsy showed no evidence of an accessory spleen In 1942 one of us studied a patient<sup>17</sup> with acquired hemolytic anemia who exhibited severe thrombocytopenia during most of seventeen weeks of hospitalization Platelet counts became normal for a short interval following splenectomy, but the thrombocytopenia recurred along with continuing hemolysis

The patient in the present series who showed clinical purpura during pregnancy seems to represent a transition between acquired hemolytic anemia and classical thrombocytopenic purpura in that the bleeding tendency and anemia were both important clinical features To complete the transition from one disease to the other we have recently studied a young woman with idiopathic thrombocytopenia who showed a relatively mild anemia which may have been caused by the persistent vaginal bleeding However, her red cells gave a positive test with the anti globulin serum in a dilution of 1-40 on several occasions Evidence of red cell sensitization ceased abruptly and completely following splenectomy, although the thrombocytopenia continued with some improvement over the course of months

The various explanations of the cause of primary thrombocytopenic purpura resolve into two principal points of view The first holds that thrombocytopenia occurs because of deficient formation of platelets in the bone marrow Dameshek and Miller (18) found an increased number of megakaryocytes in the marrow which were qualitatively abnormal in that they did not seem to be producing platelets They postulated a hormonal influence of the spleen in depressing platelet formation Excessive destruction of platelets, particularly in the spleen, is the alternative explanation for deficiency of platelets in the peripheral blood Principal exponents of this view are Doan and his co-workers, who have observed excessive phagocytosis of platelets in supravital preparations of splenic tissue<sup>19</sup> Excessive phagocytosis is also advanced as the explanation of primary splenic panhematopenia with hemolytic anemia, thrombocytopenia and leukopenia

Abnormal phagocytosis of damaged blood elements probably does occur, but we doubt if the macrophages of the intact spleen have the capacity to ingest and digest the amount of cellular elements necessary to produce a severe panhemato-

penia in view of the functional capacity of the marrow. The suggestion that an accessory spleen consisting of a few grams of tissue is capable of doing the same thing seems less plausible.

If thrombocytopenic purpura is due to the formation of an antibody-like substance similar to that found in acquired hemolytic anemia both deficient formation and excessive destruction may be important in producing the extreme degrees of thrombocytopenia sometimes observed. Sensitized platelets may be susceptible to agglutination, and phagocytosis and the presence of an anti-platelet antibody in the circulation may damage the cytoplasm of the megakaryocyte so as to inhibit the formation of platelets.

In view of the possibility of a common etiologic mechanism for acquired hemolytic anemia and thrombocytopenic purpura, it is worth noting that the available data as to the effect of splenectomy in the two diseases shows a similarity. In both conditions the effect of splenectomy is uncertain. It is usually beneficial to some degree, but in only one half to two thirds of the patients is remission complete.<sup>20-22</sup> Relapse after a remission has been observed in both groups of patients.

#### SUMMARY AND CONCLUSIONS

1. Observations of 11 patients with acquired hemolytic anemia are reported.
2. In contrast to patients with congenital hemolytic jaundice, all patients in this group exhibited evidence of sensitization of their erythrocytes by an antibody-like agent. In all patients studied there was abnormal destruction of transfused cells *in vivo*.
3. The sensitizing agent was found to be adsorbed on the erythrocytes when it could not be demonstrated in the serum. A rough method of assay of the amount adsorbed was devised by making serial dilutions of the anti-globulin serum. With this technique a fairly consistent correlation was found between the amount of antibody on the cell and activity of the disease.
4. Splenectomy when successful appears to exert a curative effect by sharply reducing the amount of antibody substance on the cell. Patients who had not responded to splenectomy in the past showed evidence of saturation of their cells with adsorbed antibody. The erythrocytes of patients who had responded to splenectomy and were in remission when studied showed distinctly less antibody on the cell by the same technique.
5. Two patients were observed to enter spontaneous remission after a long period of activity. The onset of remission in both was associated with a decrease in the amount of adsorbed immune body. However, one patient has shown evidence of return of antibody production without immediate recurrence of the hemolytic anemia. This inconsistency is not explained.
6. The tendency toward spherocytosis as measured by increased osmotic fragility may or may not be present in acquired hemolytic anemia. Prior to splenectomy the most marked increase in hypotonic fragility was observed in the patient with the most active disease. Continued activity of the disease following splenectomy was

productive of the most extreme increases in spherocytosis. This suggests that the spherocytic cells are removed from the circulation by the spleen.

7 Agglutination of red cells when the amount of adsorbed antibody reaches a critical level, together with such other phenomenon as stasis, spherocytosis, increased mechanical fragility and possibly phagocytosis probably explain the increased cell destruction.

8 The occurrence of definite and sustained leukopenia with neutropenia and thrombocytopenia in several patients with hemolytic disease due to an immune body agent raises questions as to the etiology of classic thrombocytopenic purpura and of splenic neutropenia. Patients have been observed who seem to represent transition forms between acquired hemolytic anemia and thrombocytopenic purpura. Abnormal immune mechanisms could account for both excessive destruction of platelets and deficient formation.

## REFERENCES

- <sup>1</sup> Dacie J V AND MOLLISON P L. Survival of normal erythrocytes after transfusion to patients with familial haemolytic anemia. *Lancet* 1 550 1943
- EMERSON C P JR SHEN S C HAM T H AND CASTLE W B. The mechanism of blood destruction in congenital hemolytic jaundice. *J Clin Investigation* 26 1180 1947
- <sup>2</sup> COOMBS R R A MOURANT A E AND REECE R R. A new test for the detection of weak and incomplete Rh agglutinins. *Brit J Exper Path* 26 55-66 1945
- <sup>3</sup> BOOKMAN L E DODD B E AND LOUTIT J F. Haemolytic icterus (acholuric jaundice) congenital and acquired. *Lancet* 1 812, 1946
- <sup>4</sup> EVANS R S DUANE R T AND BEHRENDT F. Demonstration of antibodies in acquired hemolytic anemia with anti human globulin rabbit serum. *Proc Soc Exper Biol & Med* 64 372, 1947
- <sup>5</sup> NEBER J AND DAMESHEK W. The improved demonstration of circulating antibodies in hemolytic anemia by the use of a bovine albumin medium. *Blood* 2 371 1947
- <sup>6</sup> STURGEON P. A new antibody in serum of patients with acquired hemolytic anemia. *Science* 106 293 1947
- <sup>7</sup> EVANS R S. Chronic hemolytic anemia. Observations of the effect of fat content of the diet and multiple red cell transfusions. *Arch Int Med* 77 544 1946
- <sup>8</sup> — DUANE R T. Observations on the effect of irradiation in chronic acquired hemolytic anemia exhibiting hemolytic activity for transfused erythrocytes. *Blood* 2 72-84 1947
- <sup>9</sup> WAOLEY P F SHEN S C GARDNER F H AND CASTLE W B. Studies on the destruction of red blood cells VI. The spleen as a source of a substance causing agglutination of the red blood cells of certain patients with acquired hemolytic jaundice by an anti human serum rabbit serum (Coombs serum). *J Lab & Clin Med* 33 1197 1948
- <sup>10</sup> STATS D ROSENTHAL, N AND WASSERMAN L R. Hemolytic anemia associated with malignant diseases. *Am J Clin Path* 17 August 1947
- <sup>11</sup> LOUTIT J F AND MOLLISON P L. Haemolytic icterus (acholuric jaundice) congenital and acquired. *J Path & Bact* 58 711 1946
- <sup>12</sup> HAM T H AND CASTLE W B. Relation of increased hypotonic fragility and of erythroblastosis to the mechanism of hemolysis in certain anemias. *Tr A Am Physicians* 55 127 1940
- <sup>13</sup> WISEMAN B K AND DOAN C A. Primary splenic neutropenia. A newly recognized syndrome closely related to congenital hemolytic icterus and essential thrombocytopenic purpura. *Ann. Int Med* 16 1097 1942.
- <sup>14</sup> ROGERS M H AND HALL B E. Primary splenic neutropenia. *Arch Int Med* 75 192, 1945
- <sup>15</sup> FISHER J A. The cryptogenic acquired hemolytic anemias. *Quart J Med* 16 45 1947
- <sup>16</sup> EVANS R S. Acute hemolytic anemia with autoagglutination. A case report. *Stanford M. Bull* 1 August 1943

- <sup>18</sup> DAMESHEK W, AND MILLER, E B The megakaryocytes in idiopathic thrombocytopenic purpura a form of hypersplenism *Blood* 1 27, 1946
- <sup>19</sup> DOAN, C A AND WRIGHT C S Primary congenital and secondary acquired splenic panhematopenia *Blood* 1 10 1946
- <sup>20</sup> WINTROBE, M M, HANRAHAN E M, JR, AND THOMAS C B Purpura haemorrhagica with special reference to course and treatment *J A M A* 109 1170 1937
- <sup>21</sup> WISEMAN, B K DOAN, C A AND WILSON, S J Present status of thrombocytopenic purpura with special reference to diagnosis and treatment *J A M A* 115 8 1940
- <sup>22</sup> VAUGHAN J M Treatment of thrombocytopenic purpura *Brit M J* 2 842-845, 1937
- <sup>23</sup> RATHER, L J *Arch Int Med* In Press
- <sup>24</sup> DAMESHEK, WILLIAM AND ESTREY S Spleen and Hypersplenism New York Grune & Stratton, 1948

# SEROLOGIC METHOD OF PURIFYING BLOOD GROUP A SUBSTANCE

By WILLIAM C. BOYD, PH D., AND ROSE M. REGUERA, B S

THE USE OF blood group A and B substance for the conditioning <sup>1</sup> of group O blood for transfusion (i.e., partially neutralizing the anti-A and anti-B agglutinins in the group O plasma) has today acquired considerable importance, and would probably continue to be used in time of war <sup>18</sup> The A substance used for this purpose has been prepared from hog stomach linings, and the B substance from horse stomachs (this B substance having also some A activity) Representative methods of preparation are described by Kabat <sup>10</sup>

Regarding any material to be added to blood before it is used for transfusion, the question of purity is, of course, important When the use of these A and B substances was first introduced, little was known about their chemical composition, although information suggesting their safety was available And, in fact, the work of Kabat <sup>9</sup> has since shown that the early materials were certainly mixtures, containing some active and some inactive material It was felt at the time that the preparation of a material known to be 100 per cent pure, even in small amounts, would be worth while, as it would provide a standard of potency with which materials offered for large scale use could be compared

It is, of course, possible that chemical methods alone could yield material which would be completely pure and 100 per cent reactive, and some of Kabat's later work suggests that he has come very close to achieving this aim Nevertheless, it seemed desirable to test an entirely different method of preparation, one in which the method of purification was primarily serologic The present communication deals with the results obtained by application of this method

It has long been known <sup>16</sup> that it is often possible, by injecting rabbits with human erythrocytes of blood group A, to obtain precipitating antibodies for A substance It was shown <sup>3, 10</sup> that such antibodies would precipitate the A substance prepared from hog stomachs It is obvious that this affords a delicate and specific method of separating serologically active A substance from inactive carbohydrates which are present and which are similar in their chemical properties If the precipitate which results when a crude preparation of group A substance and a precipitating antibody produced by the injection of human group A erythrocytes is washed thoroughly with saline, it will contain only serologically active A substance, anti-A precipitin (a modified rabbit globulin), and possibly traces of lipids and various components of complement <sup>8</sup> Removal of the antibody should leave a group A substance of a high degree of purity

## METHODS

<sup>1</sup> *Production of antisera* Rabbits were injected three times weekly with one cc of a 30 per cent suspension in saline of washed human erythrocytes of group A To avoid possible variations due to indi-

From the Department of Biochemistry Boston University School of Medicine Boston Mass  
This investigation was supported by a research grant from the Division of Research Grants and Fellowships of the National Institute of Health U S Public Health Service

vidual differences in blood group antigens the cells of a single individual (WCB) were always employed. Bleedings showed that 15 out of 20 of the animals had produced group-specific precipitins within five weeks after the injections were started. The preliminary tests were done by the interfacial technic using crude A substance. The pooled active sera were assayed for antibody content by the method of optimal proportions.<sup>8</sup> The results indicated a content of antibody nitrogen varying from 0.2 to 1.0 mg. per cc.

2. *Source of A substance.* The A substance used was a preparation of the Eli Lilly Company, designated as intended for laboratory work only (Lot numbers R 15 and 330). Analytical data for the latter preparation are given by Kabat.<sup>9</sup>

3. *Preparation of specific precipitates.* After determination of the optimal proportions ratio of a given pool of serum (previously filtered) a solution of A substance containing the calculated optimal amount plus a 10 per cent excess was added. The mixture was allowed to stand in the icebox overnight and the precipitate centrifuged off and washed until the washings were free from protein as judged by the absence of opalescence when saturated with picric acid. From three to five washings were usually required.

4. *Treatment to remove the antibody.* Various treatments designed to remove the antibody from the antibody antigen complex were tried using a total of 18 samples of crude blood group A substance totalling 362.5 mg. They included (a) digestion with trypsin (b) digestion with chymotrypsin (c)

TABLE 1.—Typical Inhibition Test. Comparison of activity of Lilly 1 per cent A Substance and Papain plus phenol treated A Anti A Precipitate

Results of tests with A<sub>1</sub> cells

Anti A 150 immune serum plus	Saline control	Dilution of solution of A substance						
		Undil.	1:10	1:10 <sup>2</sup>	1:10 <sup>3</sup>	1:10 <sup>4</sup>	1:10 <sup>5</sup>	1:10 <sup>6</sup>
1% A substance		—	—	—	—	—	+1	+
0.1% treated A Anti A precipitate	+4	—	—	—	+1	+2	+	+3

The symbol — indicates a negative reaction +1, +2, etc. indicates positive reactions of different strength +4 being complete (solid) agglutination.

digestion with papain activated with cysteine hydrochloride<sup>11</sup> (d) treatment in the Waring Blender with chloroform and amyl alcohol<sup>12</sup> (e) treatment at room temperature with 90 per cent phenol<sup>13</sup> (f) treatment at 100 C. with 90 per cent phenol (g) treatment with 0.25 N trichloroacetic acid (h) subjection to pressures of 9000 atmospheres<sup>4</sup> for twenty-four hours (i) digestion with papain followed by treatment with half-saturated ammonium sulfate or by treatment with 12 per cent sodium sulfate or by treatment with trichloroacetic acid or by treatment with 90 per cent phenol (j) denaturation by heating to 100 C. for one hour followed by papain digestion (k) heating to 55 C. in ethylene glycol. After treatment by the above methods the material was precipitated by the addition of an excess of alcohol, centrifuged, the soluble material taken up in saline or water, reprecipitated and redissolved in saline. The solution was then tested for A activity by the inhibition technic<sup>14</sup> using the original material for a control (see table 1).

## RESULTS

None of these methods, unfortunately, yielded a product which was any more active than the starting material and at the same time completely soluble in saline. Methods (i) (papain followed by phenol) and (h) (high pressure) were, on the whole, the most successful in denaturing the antibody and releasing an active antigen. Nevertheless, the resulting products were never more active than the starting material, and were often less soluble in saline. The phenol method uniformly gave products insoluble in saline, which were, moreover, only 1/100 as active as the starting material. Method (i) (papain followed by phenol) gave



product which was soluble with some difficulty, but only 1/10 as active as the starting material. The other methods were even less successful, for the resulting products were either inactive or insoluble or both.

### DISCUSSION AND CONCLUSIONS

At the time this work was begun, no estimate was possible of the percentage of the crude A substance which was specifically active. It was provisionally (and, as it proved, incorrectly) estimated that the active material did not amount to over 5 per cent at the most. If this had been correct, it is likely that one or more of the above methods would have resulted in a significant degree of purification. From Kabat's<sup>9</sup> later results, however, it is now apparent that over half of the material was serologically active, and consequently that no great degree of purification, from the serologic point of view, remained to be accomplished. Kabat's work also suggests that chemical methods have been equal to the task of producing material nearly or perhaps quite serologically pure.

The decreased solubility of the A substance after it had been precipitated with antibody and subjected to the above treatments could possibly be explained by the assumption that some of the polar groups of the A substance remained in combination with fragments, of undetermined size, of the antibody, since none of the treatments rendered it less soluble, when applied to solutions of A substance directly.

It would seem that none of the above methods offer an ideal solution to the problem of completely eliminating the antibody from a compound of antibody and antigen, even though the blood group A substance, used in these experiments, is chemically much more stable than most antigens. The converse problem, of removing *some* relatively pure antibody from an antibody-antigen compound, leaving some insoluble antibody-antigen compound to be discarded, is obviously much easier, and has been solved for several systems by various workers.<sup>6, 7, 11, 14</sup>

### REFERENCES

- <sup>1</sup> BENOICH A, KABAT E A AND BEZER, A E. Immunochemical studies on blood groups III. *J Exp Med* 83: 485-497, 1946.
- <sup>2</sup> BOIVIN A, MESAOREANU I AND MESROBEANU L. Technique pour la préparation des polysaccharides microbiens spécifiques. *Compt rend soc de Biol* 113: 490-492, 1933.
- <sup>3</sup> BOYD W C AND WARSHAWER E R. Serological relationship of blood group A and B antigens: production of anti B precipitins. *J Immunol* 50: 101-106, 1945.
- <sup>4</sup> BRIDGMAN P W. *The Physics of High Pressure*. London: G Bell & Sons Ltd, 1931.
- <sup>5</sup> DEAN H R AND WEBB R A. The influence of optimal proportions of antigen and antibody in the serum precipitation. *J Path & Bact* 29: 473-492, 1926.
- <sup>6</sup> FELTON L D. Dissociation of the specific protein precipitate of antipneumococcus horse serum and a comparison with a protein isolated by chemical means from this immune serum. *J Immunol* 22: 453-467, 1932.
- <sup>7</sup> HEIDELBERGER M AND KENDALL F E. Quantitative studies on antibody purification I. *J Exp Med* 64: 161-172, 1936.
- <sup>8</sup> — AND MAYER M. Quantitative studies on complement or alexin IV. *J Exp Med* 75: 285-295, 1942.
- <sup>9</sup> KABAT E A, BENDICH A AND BEZER A E. Immunochemical studies on blood groups II. *J Exp Med* 83: 477-484, 1946.

- <sup>10</sup> KABAT E A AND BEZER A E Immunochemical studies on blood groups I J Exp Med 82 107-215 1945
- <sup>11</sup> KALMANSON G M AND BROSTEN-BRENNER J Restoration of activity of neutralized biologic agents by removal of the antibody with papain J Immunol 47 387-407 1943
- <sup>12</sup> KLENDISHOJ N C AND WITEBSKY E Transfusion of O blood conditioned by the addition of blood group specific substances J A M A 128 1031-1033, 1945
- <sup>13</sup> MORGAN W T J, AND KING H K Studies in immunochemistry 7 Biochem J 37 640-651 1943
- <sup>14</sup> NORTROP J H Purification and crystallization of diphtheria antitoxin J Gen Physiol 25 465-485 1942.
- <sup>15</sup> SCHIFF F Uber gruppenspezifische serumpraeipitine Klin Wchnschr 3 679-680 1924
- <sup>16</sup> — AND BOYD W C Blood Grouping Technic New York Interscience Publishers Inc 194—
- <sup>17</sup> SEVAG M G LACKMAN D B AND SMOLENS J The isolation of the components of streptococcal nucleoproteins in serologically active form J Biol Chem 124 425-436 1938
- <sup>18</sup> TEDALL, L. H, GARLAND D M. AND WIENER A S A critical analysis of the value of the addition of A and B group-specific substances to group O blood for use as universal donor blood J Lab & Clin. Med 31 437-443 1946

# HEMOLYTIC REACTIONS PRODUCED IN DOGS BY TRANSFUSION OF INCOMPATIBLE DOG BLOOD AND PLASMA

## I SEROLOGIC AND HEMATOLOGIC ASPECTS

By LAWRENCE E. YOUNG, M D, DONALD M. ERVIN, M D, AND  
CHARLES L. YUILE, M D

HEMOLYTIC transfusion reactions occur more frequently than is generally appreciated and their incidence can be expected to increase for some time as the distribution of blood is facilitated. Many are doubtless overlooked because the outward manifestations may not be particularly striking, especially in anesthetized patients and in certain recipients transfused with plasma or with blood from universal donors. Despite the increasing importance of such reactions, their pathologic physiology remains poorly understood and cannot be adequately explored in human subjects. Consideration of these facts stimulated the authors to make observations on planned hemolytic reactions in dogs with the hope that the results might find general application in the field of immuno-hematology, and that they might throw light on the behavior of the kidney when subjected to certain types of insult.

The purpose of this paper is to describe preliminary serologic and hematologic observations on reactions produced in dogs by transfusion of incompatible whole blood and plasma. Typical experiments are cited to illustrate the usefulness of iso-immune systems in the dog in making quantitative studies of hemolytic phenomena. Alterations in renal physiology observed during these experiments are described in an accompanying report.<sup>1</sup>

## HISTORICAL

### *Individual Differences among Bloods of Mammals other than Dogs*

In 1900 Ehrlich and Morgenrath<sup>2</sup> found that when one goat was injected with the blood of another goat immune isolytins developed and by using such iso-immune serum a number of varieties of goat blood could be differentiated. Since that time other investigators have employed similar methods in demonstrating individual differences in the blood of other mammals. <sup>3</sup> Ottenberg and Thalheimer<sup>4</sup> demonstrated immune iso-antibodies in the serum of repeatedly transfused cats and these authors described the course of events during hemolytic reactions following injections of incompatible whole cat blood. Their findings included hemoglobinemia, hemoglobinuria, oliguria, glycosuria, hemoglobin casts in the urine, jaundice, erythrophagocytosis and leukocytosis.

### *Individual Differences among Dog Bloods*

In 1910 von Dungern and Hirschfeld<sup>5</sup> used iso immune sera to distinguish two agglutinogens and four groups among dog bloods but their observations and the few described by other investigators since 1910 have by no means completely clarified the pattern of individual differences in this species.

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From the Departments of Medicine and Pathology, The University of Rochester School of Medicine and Dentistry Rochester, N. Y.

The studies described in this report were conducted under a contract between the University of Rochester and the Office of Naval Research. This paper was presented in abridged form at the Congress of the International Society of Hematology, Buffalo, New York, August 26, 1948.

Ottenberg, Kaliski and Friedman<sup>6</sup> demonstrated what appeared to be naturally occurring iso-hemagglutinins and hemolysins in dogs. The reactions described were for the most part weak and variable but it is nevertheless significant that potent iso-hemolysins developed after repeated transfusions of but it is nevertheless significant that potent iso-hemolysins developed after repeated transfusions of agglutinable cells and not after transfusions of non agglutinable cells. Dogs whose serum appeared to contain naturally occurring iso-antibodies were transfused with apparently incompatible whole blood by use of the direct artery to vein technique. Hemolytic reactions were observed after repeated transfusions but in only one instance did such a reaction occur after the first transfusion. The recipient in this case had previously been used as a donor and it is possible that this dog was immunized by cells which entered its circulation to some extent while the artery to vein anastomosis was intact. The sequelae of incompatible transfusions in dogs were similar to those observed by Ottenberg and Thalhimer<sup>4</sup> in cats. A finding of considerable interest was the appearance of many nucleated and polychromatophilic red blood cells which in one case persisted in the recipient's circulation for five weeks. This change was attributed to the toxic effect of incompatible blood on the bone marrow.

Melnick, Burack and Cowgill<sup>7</sup> and Melnick and Cowgill<sup>8</sup> found iso-hemagglutinins and hemolysins in the serum of dogs after repeated injections of erythrocytes during the course of plasmapheresis experiments. The immune iso-antibodies reacted with red cells from about 50 per cent of the dogs available to these observers and the reactions did not appear to be related to sex or breed. In their experience the development of incompatibility was one sided in that dogs designated by them as type A were capable of producing antibodies against cells from dogs of type B while B dogs did not produce antibodies when transfused with A cells. Salivation, vomiting, labored respirations, incontinence of urine and feces, prolonged clotting time and hemoglobinuria were observed in immunized A dogs during hemolytic reactions to the transfusion of B cells.

Holman, Mahoney and Whipple<sup>9</sup> and Wright<sup>10</sup> described similar reactions as complications of plasmapheresis and Wright found antibodies in the sera of 3 recipient dogs which reacted with cells of 7 members of a group of 11 donor dogs. Hahn and Bale<sup>11</sup> also encountered hemolytic reactions while measuring circulating red cell mass in dogs by transfusion of cells tagged with radioactive iron. They found moreover, that all of the new isotopic cells if incompatible, usually disappeared from the circulation of the recipient dog within ten minutes after transfusion. Miller, Robscheit Robbins and Whipple<sup>12</sup> observed hemolysis of recipient dogs' cells following transfusions of plasma from a dog that might have been immunized by previous injections of dog blood.

## METHODS

Dog iso-antibodies were titrated by mixing equal volumes (usually 0.1 ml.) of serial two-fold dilutions of serum with 5 per cent suspensions of dog cells in fresh unheated autologous serum. Immune serum was inactivated by heating at 56°C. for thirty minutes and was routinely diluted with saline, since it was found that titers against cells suspended in unheated serum were invariably the same regardless of whether the antiserum was diluted with saline or with normal dog serum. Titers were maximal when fresh normal dog serum was used either as a suspension medium for the cells or as a diluent for the antiserum and were not further enhanced by employing normal serum both as a medium for suspending cells and as a diluent for the antiserum.

After standing fifteen minutes at room temperatures of 23-27°C. the tubes were centrifuged at 1000 RPM for one minute. The cells were then gently but thoroughly resuspended and examined over a well illuminated concave mirror. Titers were expressed in terms of the final dilution of antiserum in the last tube showing agglutination. Agglutinated cells were rapidly hemolyzed in tubes containing high concentrations of antibody and complement but in the last tubes of any given series the agglutinated cells seldom hemolyzed appreciably during the fifteen minute period. Nonspecific hemolysis was minimized by carrying out the titrations at room temperature rather than at 37°C. and by centrifuging the tubes after allowing them to stand for a relatively short period (fifteen minutes).

\* The ability of certain dog antisera to agglutinate dog erythrocytes is enhanced by the presence of a heat labile component of normal dog serum as described in a separate report in the literature. This has thus far been observed only in sera having so-called anti-D or canine A antibodies. The heat labile component of complement has thus far been found only in sera having so-called anti-D or canine A antibodies. The heat labile component of complement has thus far been found only in sera having so-called anti-D or canine A antibodies.

† Only D or canine A antibodies have thus far been found to enhance the hemolytic activity of complement. Other dog isoantibodies that have been tested have not been found to enhance the hemolytic activity of complement.

*Complement* was measured by mixing serial two-fold dilutions of fresh dog serum in volumes of 0.3 ml with 0.2 ml volumes of sensitized sheep cells prepared by Wadsworth's<sup>14</sup> method. The tubes were placed in a water bath maintained at 37 C, shaken at five minutes and examined for hemolysis at fifteen minutes. The 50 per cent end point was then computed by the method of Heden<sup>15</sup> which takes into account the degrees of hemolysis in the last four tubes showing reaction.

All *transfused blood* was drawn from normal donor dogs within one hour prior to the beginning of transfusion and was injected into one of the jugular veins of the unanesthetized recipient dogs at rates of 3 to 7 ml per minute. A saturated solution of sodium citrate (1.0 ml per 100 ml of blood) was used as an anticoagulant in all but two transfusions and in these two instances heparin was employed. Samples of blood from recipient dogs were drawn from the jugular veins with great care to minimize artificial hemolysis. It was found advantageous to coat the inner surfaces of syringes and needles with silicone in order to prevent coagulation during the withdrawal and delivery of large samples into multiple containers.

The concentration of *hemoglobin* in plasma was measured by the pyridine hemochromogen method of Flink and Watson<sup>16</sup> and bilirubin was quantitated by Ducci and Watson's<sup>17</sup> modification of the method of Malloy and Evelyn.<sup>18</sup> *Osmotic fragility of erythrocytes*,<sup>19</sup> *coagulation time of whole blood*<sup>20</sup> and *prothrombin concentration*<sup>21, 22</sup> were determined by procedures described elsewhere. *Platelets* were enumerated according to Wintrobe's<sup>23</sup> description of the Rees-Ecker technic. *Differential agglutination of dog erythrocytes* (Ashby technic) was carried out by the method of Young, Platzer and Rafferty.<sup>24</sup>

*Reticulocytes* were stained by mixing a small drop of oxalated blood on a glass cover slip with a large drop of 0.2 per cent suspension of brilliant cresyl blue in 0.6 per cent solution of sodium chloride. The two drops were mixed for thirty seconds with a toothpick, after which time another cover slip was applied and smears were pulled, dried and counterstained with Wright's stain to make permanent preparations.

Blood used for *enumeration of leukocytes* and for preparation of Wright's stained smears on glass cover slips was taken from a small incision in the marginal ear vein and was used without addition of any coagulant. Smears thus prepared were employed for *differential leukocyte counts* and were routinely examined for the presence or absence of spherocytosis and erythrophagocytosis. Both glass and plastic cover slips were used in making wet preparations of oxalated, defibrinated or heparinized venous blood to be examined for the presence or absence of *spherocytosis*, *erythrophagocytosis* and *hemagglutination*.

## EXPERIMENTAL OBSERVATIONS

### *Definition of Do-positive and Do-negative Dogs*

Our studies began with the demonstration of immune iso-hemagglutinins and hemolysins in the serum of a dog that had had a hemolytic reaction after a series of transfusions from several donors. Serum from this dog agglutinated and hemolyzed erythrocytes from about two-thirds of the dogs selected at random from the animal colony maintained by the University of Rochester School of Medicine and Dentistry.\* Cells reacting with this serum, or subsequently with other dog sera having similar specificity, were tentatively labelled *Do-positive*, while those that were neither agglutinated nor lysed were called *Do-negative*.

Further serologic studies on more than 400 dogs indicate that, in addition to the *Do* factor, there are at least three other antigenic factors present in various combinations in dog erythrocytes. The antigenic structure of canine red cells has

\* The assistance of Dr. F. S. Rohnsheit Robbins, Dr. Paul Rekers, Dr. Herbert Stokinger and others in the collection of specimens of dog blood is gratefully acknowledged. The previously cited observations of Holman, Mahoney and Whipple,<sup>9</sup> Wright,<sup>10</sup> Hahn and Bale,<sup>11</sup> and Miller, Rohnsheit Robbins and Whipple<sup>12</sup> were made in the Department of Pathology of the University of Rochester School of Medicine and Dentistry. Their experiences were to a considerable extent responsible for the decision of the authors to carry out the studies reported in this paper.

not yet been determined to our satisfaction but is now being explored more extensively and will be the subject of a later report. Our attention has until recently been devoted for the most part to the study of hemolytic transfusion reactions due to Do antibodies.

### *Iso-immunization of Dogs*

The immunization programs in three typical experiments are illustrated in figure 1. The top graph shows that antibodies were first detected eleven days after a single large transfusion of Do-positive blood into a Do-negative dog that had not been previously transfused. Rapid disappearance of the donated cells at the

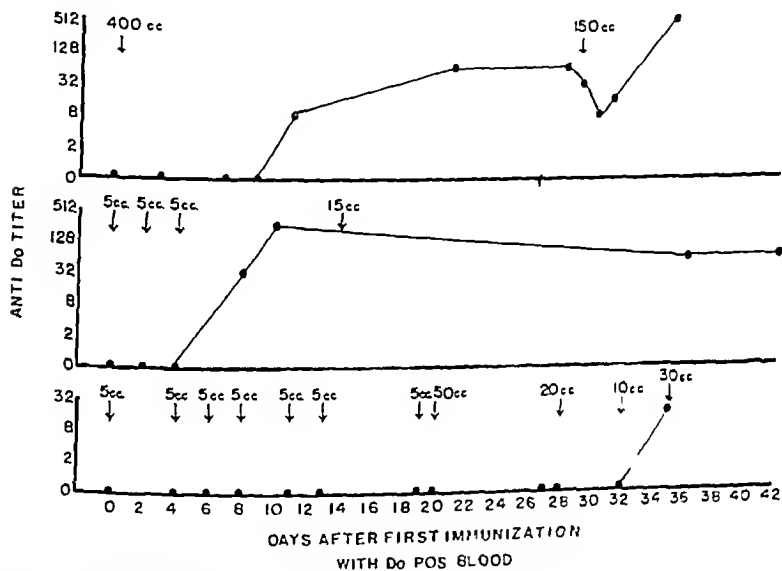


FIG. 1—TYPICAL EXPERIENCES WITH IMMUNIZATION OF DO-NEGATIVE DOGS BY INTRAVENOUS INJECTION OF DO-POSITIVE WHOLE BLOOD

time of antibody development was demonstrated with the Ashby technique by employing potent anti-Do serum. In the middle graph it can be seen that three small injections produced antibodies within eight days in a dog previously transfused with dog bloods of unknown type. The bottom graph shows that an antibody developed only after 10 injections over a period of thirty-five days in a relatively refractory dog that had not been previously transfused. Still more refractory dogs have recently been encountered. For example, one dog developed an antibody only after 17 injections had been given over a period of one hundred thirty days.

### *Characteristics of Dog Iso-antibodies*

Do iso-antibodies have been found to fix complement both in vitro and in vivo, and it has been repeatedly observed that in the presence of complement

nated Do-positive cells are subsequently hemolyzed *in vitro* at rates depending upon the amounts of antibody and complement present. The Do and Rh systems in the dog and human species respectively appear to have a number of features in common. An important difference, however, is that Do-antibodies hemolyze Do-positive cells relatively quickly in the presence of complement while Rh antibodies hemolyze Rh-positive human cells very slowly if at all.<sup>26</sup> Under certain conditions Do-antibodies behave like incomplete Rh antibodies in that their attachment to erythrocytes can be demonstrated by developing tests employing anti-dog-serum rabbit serum and by the agglutination of sensitized cells when suspended in normal dog serum. Characteristics of dog iso-antibodies will be described in more detail in a separate report.<sup>12</sup>

### *Observations on Hemolytic Transfusion Reactions*

Serial observations have thus far been made during the course of twenty-three hemolytic reactions produced in 13 different recipient dogs by transfusion of

TABLE 1—*Prominent Manifestations Observed during the Course of Twenty-three Hemolytic Transfusion Reactions Produced in 13 Different Recipient Dogs*

Restlessness	} Nearly 100 per cent
Salivation	
Vomiting	
Incontinence	
Fever	} Variable
Shock	
Hives	
Immediate death	
	3 dogs
	1 dog

incompatible whole dog blood or plasma. Prominent manifestations observed during the periods immediately following transfusion are recorded in table 1 which, for purposes of the present discussion, requires no further comment.

### *Transfusion of Incompatible Whole Blood*

Figure 2 illustrates the manner in which the concentrations of hemoglobin and bilirubin rose and fell in the plasma of a Do-negative recipient after typical transfusions of Do-positive whole blood. When the recipient's anti-Do titer was 1:256 the peak of hemoglobinemia was nearly twice as high after a transfusion of only 100 ml of Do-positive blood as it was after a transfusion of 200 ml from the same donor into the same 15 kilogram recipient at an earlier date when the anti-Do titer was only 1:2. The hemoglobinemia curve was flatter when the recipient's titer was low and the volume of transfused blood was large. The less rapid destruction of donated cells *in vivo* under these circumstances was in keeping with the results of *in vitro* experiments. The concentration of hemoglobin in the plasma was nevertheless maximal within 10 minutes after this transfusion was completed, and it was maximal, or nearly so, at 5 to 10 minutes in most of the other experiments. Bilirubinemia, on the other hand, was maximal at 3 to 6 hours after each

transfusion of incompatible whole blood and in nearly every instance the concentration of bilirubin in the plasma had returned to the normal range within 24 hours.

Observations made before and after another typical transfusion of incompatible whole blood are recorded in figure 3. In this experiment, the donated corpuscles were tagged with radioactive iron\* and it was possible to show that these cells completely disappeared from the recipient's circulation within the first hour after the transfusion was completed. In fact, 84 per cent of the donated erythrocytes disappeared within 10 minutes after completion of the transfusion, or within 30 minutes after its start. In four other experiments employing tagged cells, nearly all of the donated erythrocytes disappeared within 30 to 90 minutes after com-

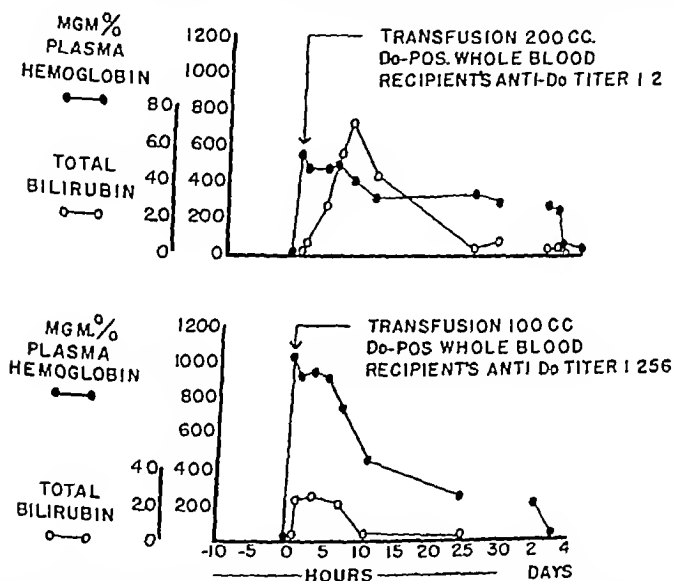


FIG. 2.—RELATIONSHIP OF HEMOGLOBINEMIA AND BILIRUBINEMIA TO ANTIBODY TITER AND VOLUME OF TRANSFUSION. The same Do-positive donor and immunized Do-negative recipient were used in both of these transfusions.

pletion of the transfusions. It is therefore little wonder that the hemoglobinemia curves differed so slightly from those obtained after intravenous injection of hemoglobin solutions, and that significant morphologic changes could not be detected in smears or wet preparations made from venous blood of the recipients. The very rapid disappearance of donated cells also explains the observation that only a barely measurable portion of the erythrocytes present in the recipient's circulation after such transfusions showed slightly increased osmotic fragility.

Surprisingly rapid disappearance of incompatible cells was further demonstrated

\* Drs. James A. Bush, John W. Hayden and Henry Tesluk assisted with the measurements of activity which were made by a modification of the donor cell dilution method of Bush and volume.



by using the Ashby technic after this transfusion and in six other similar experiments. In no case could agglutinable Do-positive cells be demonstrated after transfusion by mixing potent anti-Do serum with samples of recipient's blood. When the Ashby method was applied to the Do-anti-Do system after transfusion of compatible cells, on the other hand, donated Do-negative corpuscles were shown to survive for at least three months in the circulation of a Do-positive dog. This observation on the life span of canine erythrocytes is in accord with estimates made by other methods.

The amount of complement present in the circulation of the recipient dog declined abruptly during this transfusion of incompatible whole blood. In each of 10

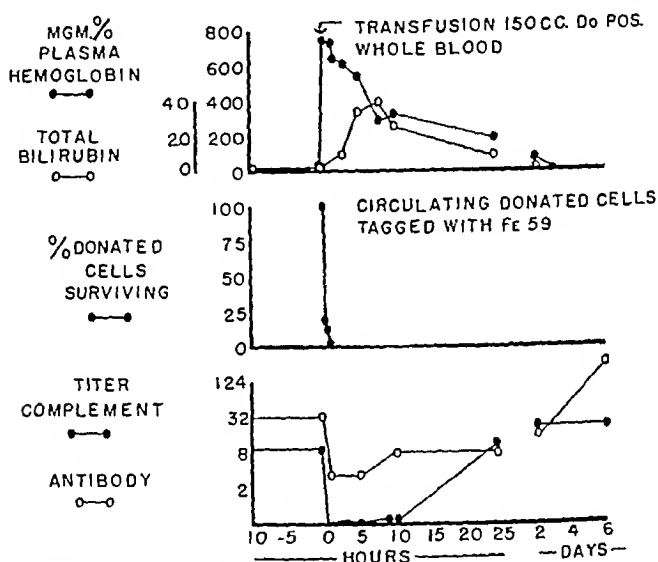


FIG. 3.—SEQUELAE OF TYPICAL TRANSFUSION OF DO-POSITIVE WHOLE BLOOD INTO IMMUNIZED DO-NEGATIVE RECIPIENT (WEIGHT 15 KG.)

other similar experiments the decline was equally precipitous and after large transfusions, complement was barely detectable for about five hours. Post-transfusion specimens of serum were not anticomplementary, despite their high content of free oxyhemoglobin. At twenty-four hours and for several days thereafter, the titer of complement was frequently higher than before transfusion. The fall and subsequent rise in antibody titer noted after this transfusion were observed in some of the other experiments but with much less regularity than the changes in concentration of complement.

Fluctuations in total and differential nucleated cell counts after the typical transfusion just referred to are recorded in figure 4. The transient leukopenia, followed by leukocytosis, shift to the left and a shower of nucleated red cells, was observed after nearly all injections of incompatible whole blood. Erythrophago-

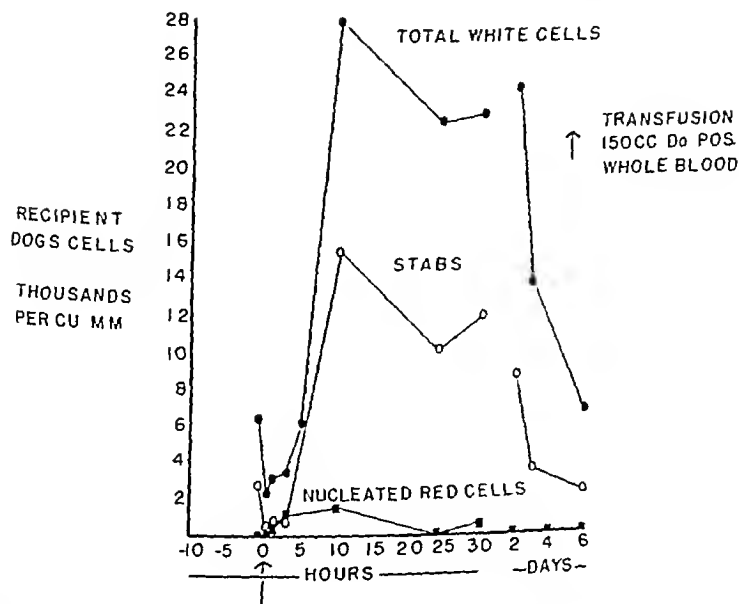
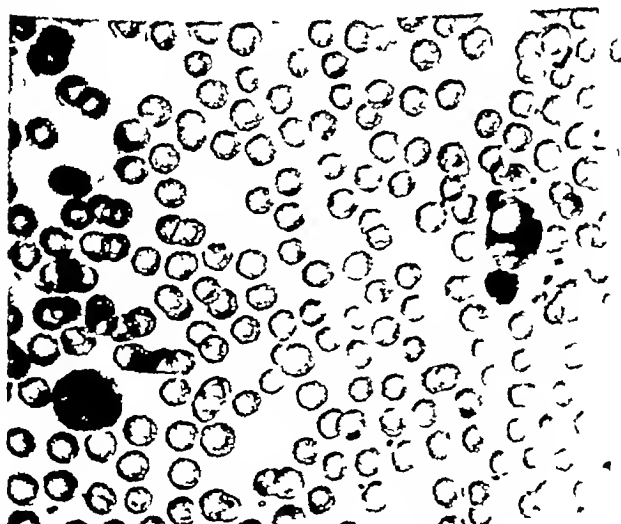


FIG 4.—FLUCTUATIONS IN TOTAL AND DIFFERENTIAL COUNTS OF NUCLEATED CELLS IN CIRCULATION OF IMMUNIZED DO-NEGATIVE RECIPIENT AFTER SAME TRANSFUSION PLOTTED IN FIG 3



cytosis (fig 5) was observed to a slight extent in smears of venous blood prepared during the first few minutes after such transfusions, but it was always necessary to search many microscopic fields before finding macrophages containing red cells. Wet preparations proved to be less satisfactory than fixed smears for detection of erythrophagocytosis. In none of the wet preparations of venous blood from recipient dogs was hemagglutination (suggesting intravascular agglutination) observed. Platelets became slightly less numerous for a few hours after injections of whole blood, and transient increases in coagulation time and decreases in prothrombin concentration\* were observed in some instances.

Electrophoretic studies\* were carried out on samples of plasma taken before transfusion, and at 30 minutes, 4 hours and 23 hours after a transfusion of 60 ml of incompatible whole blood. The only significant change in the patterns was the appearance at 30 minutes of a large peak with a mobility between that of fibrinogen and beta globulin. The area of the peak corresponded with the concentration of hemoglobin in the plasma as determined by the pyridine hemochromogen method. Light transmitted through the cell in the region of this peak showed the absorption bands characteristic of oxyhemoglobin. At 4 hours the height and area of the peak had slightly diminished and at 23 hours the peak had almost disappeared. It is worthy of note that at 30 minutes and at 4 hours the hemoglobin migrated with an abnormally low mobility, but at 23 hours the mobility of hemoglobin had returned to normal.

Nearly all post-transfusion specimens of plasma were examined with a hand spectroscope in an effort to detect the presence of methemoglobin or methemalbumin. The absorption bands were invariably those of oxyhemoglobin, absorption in the red portion of the spectrum was not observed.

The concentrations of sodium and potassium in the serum were not significantly increased after transfusions of incompatible whole blood.\* These negative findings are of interest in view of the relatively high content of sodium and low content of potassium in dog erythrocytes as compared with human red cells.<sup>27</sup> Muirhead et al.,<sup>28</sup> on the other hand, have reported high concentrations of potassium in the serum of human recipients following transfusions of incompatible human cells.

### *Transfusion of Incompatible Plasma*

When Do-positive dogs were transfused with plasma from immunized Do-negative dogs the course of events was distinctly different from that seen after administration of incompatible whole blood. It is evident in figure 6 that after transfusion of 45 ml of plasma with an anti-Do titer of 1:256, the concentration of hemoglobin in the plasma of the 16 kilogram Do-positive recipient did not reach its peak until the fifth hour. Hemoglobinemia persisted for more than 72 hours and hyperbilirubinemia for more than 24 hours. In order to sustain this dog's life it was necessary to give 260 ml of compatible Do-negative whole blood 5 hours after injection of the incompatible plasma. Despite this large transfusion, the recipient dog's hematocrit gradually fell to 22 per cent on the ninth day, after which time

\* Determinations of prothrombin concentration were made by Dr. Ralph F. Jacox; electrophoretic studies by Dr. Eric Alling; and measurements of serum sodium and potassium by Dr. Jacob W. Holler.

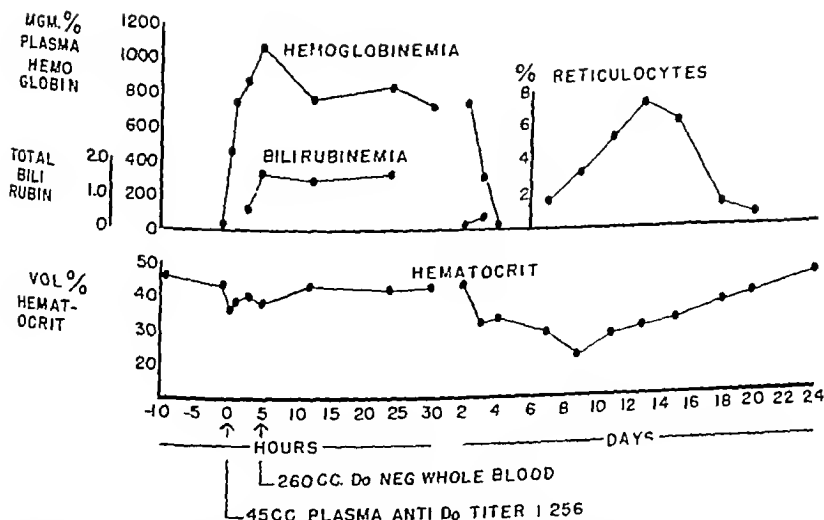


Fig 6—SEQUELAE OF TRANSFUSION OF ANTI DO PLASMA INTO DO POSITIVE RECIPIENT (WEIGHT 16 Kg) Compatible Do-negative whole blood was given 5 hours later to sustain life

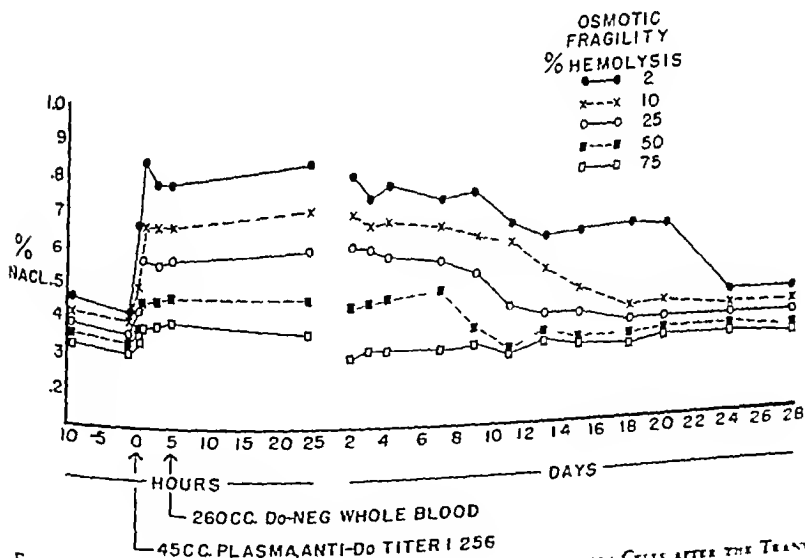


Fig 7—LATERAL PLOT OF OSMOTIC FRAGILITY OF DO-POSITIVE RECIPIENT'S CELLS AFTER THE TRANSFUSION OF ANTI DO PLASMA PLOTTED IN FIG 6

the hematocrit began to rise due to the formation of new cells. The peak of the reticulocyte response was reached on the thirteenth post transfusion day. It is of interest that there was as only a moderate decrease in the titer of complement.

during the first five hours after this transfusion of plasma and that at no time could the donated Do-antibodies be demonstrated in the recipient dog's serum. The osmotic fragility of the recipient dog's erythrocytes, as observed over a period of four weeks after transfusion of incompatible plasma, is plotted laterally in figure 7. Spherocytosis (fig. 8) and increased fragility were evident for twenty days and the period of marked increase in fragility corresponded well with the nine-day period of falling hematocrit shown in figure 6. These findings were similar to those reported by Banti,<sup>9</sup> Dameshek and Schwartz<sup>20</sup> and Tigerit and Duncan<sup>2</sup> who injected dogs and guinea pigs with immune *hetero*-antibodies produced in other species.



FIG. 8—SPHEROCYTES IN SMEAR OF VENOUS BLOOD OF DO-POSITIVE RECIPIENT SEVEN DAYS AFTER TRANSFUSION OF ANTI DO PLASMA. 1500 X

#### DISCUSSION

The prolonged destruction of recipient dogs' Do-positive cells after injection of incompatible plasma is in striking contrast to the very rapid elimination of donated Do-positive corpuscles after transfusion to immunized Do-negative recipients. In the human species, recipients' cells are likewise known to be destroyed over long periods of time after transfusions of incompatible plasma or of blood from dangerous universal donors,<sup>32-34</sup> while incompatible donated cells often disappear with relative rapidity.<sup>3, 35, 36</sup> In neither species, however, is it entirely clear how the various destructive mechanisms operate under these circumstances.

With the evidence at hand it seems likely that the very rapid elimination of donated Do-positive cells is due in large measure to intravascular hemolysis, both by the direct action of complement on sensitized cells and by the traumatic effect\* of circulation on injured and agglutinated cells. Intravascular erythrophagocytosis probably plays a very minor role.

The concentration of hemoglobin in recipient dogs' plasma is usually maximal, or nearly so, within five to ten minutes after incompatible cells are injected, but the peak may not be reached until three to five hours have elapsed. Review of the experiments thus far completed shows that the maximal plasma hemoglobin mass, calculated on the basis of highest plasma hemoglobin concentration and estimated blood volume, is in each case equivalent to approximately 50 to 75 per cent of the hemoglobin contained in the transfused incompatible cells. In estimating the total amount of hemoglobin liberated intravascularly, however, one must also take into account (a) hemoglobin excreted in the urine or taken up by renal tubules or by other tissues prior to the moment at which maximal plasma hemoglobin concentration is reached, and (b) hemoglobin liberated intravascularly after the concentration in the plasma reaches its peak.

Data thus far obtained therefore indicate that well over 50 to 75 per cent of the cells that rapidly disappear from the recipient's circulation are destroyed intravascularly and that a relatively small proportion of the cells may be sequestered and destroyed extravascularly by the reticulo-endothelial system. This conjecture is based upon the assumption that hemoglobinemia is the result of intravascular hemolysis and that hemoglobin liberated from erythrocytes by reticulo-endothelial cells is converted to bilirubin before being released into the blood stream.<sup>2</sup> In any event, when anti-Do plasma is transfused, the relative importance of the several destructive mechanisms may be quite different from that encountered after injection of incompatible cells.

Experiments in progress<sup>36</sup> should demonstrate more precisely how dog erythrocytes are destroyed *in vivo* under a variety of conditions simulating those encountered clinically.

### SUMMARY

1. Dogs injected intravenously with dog erythrocytes containing one or more antigenic factors lacking in their own red cells developed iso-hemagglutinins and hemolysins exhibiting characteristics of immune antibodies.

2. Transfusions of incompatible whole dog blood and plasma were carried out under controlled conditions. Pretransfusion observations were made and followed by closely spaced post-transfusion measurements of serologic and hematologic alterations.

3. The rate of destruction of incompatible donated corpuscles was determined by tagging the cells with radioactive iron and also by employing the technique of differential agglutination of erythrocytes. It was thereby shown that all cells

\* Because of technical difficulties encountered in measuring mechanical factors in this aspect of the problem will be dealt with in a separate communication.

incompatible donated cells disappeared from the recipient's circulation within the first thirty to ninety minutes following transfusion. The probable mechanisms and relative importance of intra- and extravascular destruction of erythrocytes are briefly discussed.

4 Destruction of recipient dogs' corpuscles by donated immune plasma was relatively slow, and spherocytosis and increased osmotic fragility of the recipient's cells were evident for periods as long as twenty days. These observations are compared with those made in human beings after transfusions of plasma and of blood from dangerous universal donors.

5 The titer of complement in the sera of recipient dogs was sharply reduced for at least five hours after all transfusions of incompatible whole blood, but isoagglutinin titers were less regularly reduced after such transfusions.

6 Other notations of interest included estimates of the concentrations of serum bilirubin, sodium and potassium, determinations of clotting time, prothrombin concentration, and observations on red cell morphology, intravascular erythrophagocytosis, and shifts in distribution of leukocytes and in the electrophoretic patterns of plasma.

### CONCLUSION

The transfusion experiments thus far completed with dog blood are considered only exploratory. They are sufficient nevertheless to justify the conclusion that the iso-immune systems in the dog may be used to advantage in quantitative studies on certain hemolytic phenomena that cannot be satisfactorily investigated in human beings.

### ACKNOWLEDGMENTS

It is a pleasure to acknowledge the technical assistance of Mrs. Jane Peters, Miss Mary Jane Izzo and Miss Shirley Deshon.

### REFERENCES

- 1 YULE, C. L., VAN ZANDT, T. ERVIN, D. M., AND YOUNG, L. E. Hemolytic reactions produced in dogs by transfusion of incompatible dog blood and plasma. II. Renal aspects following whole blood transfusions. *Blood*, this issue, p. 1232.
- 2 EHRLICH, P., AND MORGENROTH, J. Über Haemolysine. *Berl. Klin. Wchnschr.* 37: 453, 1900.
- 3 WIENER, A. S. *Blood Groups and Transfusion*, ed. 3. Springfield, Ill., C. C. Thomas, 1943. P. 345.
- 4 OTTENBERG, R., AND THALHIMER, W. Studies in experimental transfusions. *J. Med. Research* 33: 213, 1915.
- 5 VON DUNOERN, E., AND HIRSCHFELD, L. Ueber Nachweis und Vererbung biochemischer Strukturen. *Ztschr. f. Immunitäts* 4: 531, 1910.
- 6 OTTENBERG, R., KALISKI, D. J., AND FRIEDMAN, S. S. Experimental agglutinative and hemolytic transfusions. *J. Med. Research* 28: 241, 1913.
- 7 MELNICK, D., BURACK, E., AND COWOILL, G. R. Development of incompatibilities in dogs by repeated infusion of red blood cells. *Proc. Soc. Exper. Biol. & Med.* 33: 616, 1935.
- 8 ——— AND COWOILL, G. R. Differentiation of blood groups in dogs based on antigenic complexes present in the erythrocytes. *Proc. Soc. Exper. Biol. & Med.* 36: 697, 1937.
- 9 HOLMAN, R. L., MAHONEY, E. B., AND WHITTLE, G. H. Blood plasma protein regeneration controlled by diet. I. Liver and casein as potent diet factors. *J. Exp. Med.* 39: 251, 1934.
- 10 WRIGHT, A. Isohemolysins and isoagglutinins occurring in dogs. *Proc. Soc. Exper. Biol. & Med.* 34: 440, 1936.
- 11 HAHN, P. F., AND BALE, W. F. Linear relationship between the circulating red cell mass and the venous hematocrit as determined with radioactive iron. *Am. J. Physiol.* 136: 314, 1942.

- <sup>11</sup> MILLER, L. L. ROBSCHT F ROBBINS F S AND WHIPPLE G H Hemoglobin and plasma protein—their relationship to internal body protein metabolism *J Exp Med* 81 425 1945
- <sup>12</sup> CHRISTIAN R. M. ERVIN D M, AND YOUNG L E Manuscript in preparation
- <sup>13</sup> WADSWORTH A B Standard Methods Williams and Wilkins Baltimore Md 1928
- <sup>14</sup> HEDEN C G On the estimation of fifty per cent end points in serological titrimetry *J Path & Bact* 58 477 1946
- <sup>15</sup> FLINK, E. B. AND WATSON C J A method for the quantitative determination of hemoglobin and related heme pigments in feces, urine and blood plasma *J Biol Chem* 146 171 1942
- <sup>16</sup> DUCCI H AND WATSON C J Quantitative determination of serum bilirubin with special reference to prompt-reacting and chloroform soluble types *J Lab & Clin Med* 30 293 1945
- <sup>17</sup> MALLOT H T AND EVELYN A A Determination of bilirubin with photoelectric colorimeter *J Biol Chem* 119 381 1937
- <sup>18</sup> SEEN S C HARRIS, T H AND FLEMING E M Studies on the destruction of red blood cells III Mechanism and complications of hemoglobinuria in patients with thermal burns spherocytosis and increased osmotic fragility of red blood cells *New England J Med* 229 701, 1943
- <sup>19</sup> LEE, R. I. AND WHITE, P. D A clinical study of the coagulation time of blood *Am J M Sc* 145 495 1913
- <sup>20</sup> QUICK, A. J The prothrombin in hemophilia and obstructive jaundice *J Biol Chem* 109 LXXIII 1935
- <sup>21</sup> WARNER E D BRINKHOUSE K. M AND SMITH H P A quantitative study of blood clotting Prothrombin fluctuations under experimental conditions *Am J Physiol* 114 667 1936
- <sup>22</sup> WINTROBE M. M Clinical Hematology ed 2. Philadelphia Lea and Febiger 1946 P 193
- <sup>23</sup> YOUNG L E PLATZER R F AND RAFFERTY J A Differential agglutination of human erythrocytes *J Lab & Clin Med* 32 489 1947
- <sup>24</sup> HAHN P F ROSS J F, BALE W F BALFOUR W M AND WHIPPLE G H Red cell and plasma volumes (circulating and total) as determined by radio-iron and by dye *J Exp Med* 75 221 1942.
- <sup>25</sup> HILL, J M, HABERMAN S AND JONES F Hemolytic Rh immune globulins Evidence for a possible third order of antibodies incapable of agglutination or of blocking Blood Special Issue No. (The Rh Factor) p 80 1948
- <sup>26</sup> DAVSON H, AND DANIELLI J F The Permeability of Natural Membranes New York The Macmillan Company 1943 P 139
- <sup>27</sup> MUIRHEAD S E HALEY A E HABERMAN S AND HILL, J M Acute renal insufficiency due to incompatible transfusion and other causes with particular emphasis on management Blood Special Issue No. 2 (The Rh Factor) p 101 1948
- <sup>28</sup> BANTI G Travaux originaux Splénomégalie hémolytique anhémo-poïétique la rôle de la rate dans l'hémolyse *Semaine méd* 33 313 1913
- <sup>29</sup> DAMESHEK, W, AND SCHWARTZ, S O Hemolysins as a cause of clinical and experimental hemolytic anemias *Am J M Sc* 196 769 1938
- <sup>30</sup> TIGERTT W D AND DUNCAN C. N Erythrocyte morphology in experimental hemolytic anemia as induced by specific hemolysin *Am J M Sc* 200 173 1940
- <sup>31</sup> EBERT R V AND EMERSON C. P A clinical study of transfusion reactions The hemolytic effect of group O blood and pooled plasma containing incompatible isoagglutinins *J Clin Invest* 23 617 1946
- <sup>32</sup> GIBSON J G AND AUB J C EVANS R D PEACOCK, W C IRVINE J W JR AND SACK T The measurement of post transfusion survival of preserved stored human erythrocytes by means of radioisotopes of radioactive iron *J Clin Investigation* 24 1947
- <sup>33</sup> ERVIN D M AND YOUNG L E Unpublished observations
- <sup>34</sup> YOUNG L E AND KARRER D H Hemolytic transfusion reactions due to Rh incompatibility *JAMA* 127 6-7 1945
- <sup>35</sup> PLATZER R F YULE, C L AND WOODRUFF F L Reactions of group O blood plasma with group A blood fusion of two liters of group A blood *Am J Clin Path* 1 100 1947
- <sup>36</sup> ROSS J F Hemoglobinuria and hemolysis *New England J Med* 188 111 1923
- <sup>37</sup> ERVIN D M CHRISTIAN R M DAVIS R W YOUNG L E AND YULE C L Unpublished observations



# HEMOLYTIC REACTIONS PRODUCED IN DOGS BY TRANSFUSION OF INCOMPATIBLE DOG BLOOD AND PLASMA

## II RENAL ASPECTS FOLLOWING WHOLE BLOOD TRANSFUSIONS

By CHARLES L. YUILE, M.D., THEODORE F. VAN ZANDT, A.B., DONALD M. ERVIN, M.D., AND LAWRENCE E. YOUNG, M.D.

DEATH FROM renal insufficiency with the postmortem findings of hemoglobinuric or lower nephron nephrosis frequently follows acute hemolytic reactions of various etiology including the transfusion of incompatible blood<sup>1-3</sup>. Extensive studies based largely upon the injection of solutions of hemoglobin, related pigments or laked blood as substitutes for hemolysis *in vivo*, have been carried out over a period of many years, but have failed to explain the exact mechanism of this type of renal failure<sup>3-5</sup>.

The hemolytic reactions in dogs produced by transfusion of incompatible dog blood described in the preceding paper<sup>6</sup> afford an ideal opportunity to study the effects of such reactions upon the kidney under a variety of conditions simulating those seen clinically.

It has been shown by several groups of investigators, using different animal species, that induced hemoglobinemia within the range encountered in most acute hemolytic disturbances in human subjects, produces only transient changes in normal animals with previously undisturbed renal function<sup>4, 7-10</sup>. On the other hand, particularly if the urine is acid, the injection of hemoglobin into an animal in a severe state of dehydration<sup>11</sup> or with kidneys previously injured<sup>4</sup> results in the formation of pigment casts in the renal tubules followed frequently by death in uremia. Similar results have been reported in dogs following the injection of very large amounts of hemoglobin<sup>6</sup> or laked red blood cells<sup>1</sup>.

This preliminary report is concerned with a controlled study of renal function carried out in conjunction with the experiments described in the preceding paper. The results indicate that in the *normal* dog with *either acid or alkaline* urine a combination of the intravascular hemolysis and other profound changes resulting from the transfusion of incompatible blood is not sufficient to produce renal failure.

### METHODS

Procedures used in the immunization of recipient dogs and the collection and transfusion of incompatible blood have been described in part one of this report.<sup>6</sup> All dogs were normal mongrels vaccinated against distemper. Female animals were used in all experiments involving quantitative renal function studies, urine being obtained through a curved metal catheter. For male dogs a ureteral catheter was used. Water was given by stomach tube at intervals prior to each transfusion to insure adequate urine flow.

From the Departments of Pathology and Medicine, The University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

The studies described in this report were conducted in part under a contract between The University of Rochester and the Office of Naval Research. This paper was presented in an abridged form at the International Hematology Congress, Buffalo, New York, August 26, 1948.

and sodium bicarbonate was added when alkaline urine was desired. In order to obtain the secretion of acid urine with a pH of about 6.0 or less at the time of transfusion dogs were fed a diet of horsemeat 200 Gm, lard 50 Gm and ammonium chloride 3 Gm for a period of three to four days.

Urinary hemoglobin concentration was determined by the cyanmethemoglobin method of Evelyn and Malloy.<sup>12</sup> Radioactive iron determinations<sup>14</sup> were made on the total hemoglobin excreted in those experiments in which the donated cells were labelled with this isotope.\*

Effective renal plasma flow was measured by means of *para* aminohippurate clearance (15)† Satisfactory blood and urine levels were obtained by injecting 0.5 to 0.8 ml of the drug intraperitoneally 15 minutes before each 20 minute collection period. Glomerular filtration rates were determined by measuring mannitol clearance (16)† in some experiments and creatinine clearance<sup>17</sup> in others.

TABLE 1—Summary of Fourteen Hemolytic Transfusion Reactions in Dogs in which Renal Functions were Studied

Dog number	Sex	Weight	Initial pH of urine	Blood transfused	Recipient's antibody titer	Maximal plasma Hb	Approx. period of hemoglobinuria	Total Hb excreted % of Hb transfused	Maximal blood urea nitrogen
		Kg		ml		mg per 100 ml	hours		mg per 100 ml
43 31	Female	13.2	7.5-8.0	205	1-2	535	18	10	—
43 31		13.2	7.5-8.0	100	1-256	1130	20	17	20.5
43 31		13.2	7.5-8.0	128	1-256	1180	20	27	11.5
43 31		13.2	7.5-8.0	130	1-128	1110	24	37	17.5
43 381		20.3	7.5-8.0	150	1-32	750	17	18	26.3
43 381		20.0	7.58	75	1-16	543	8	25	16.5
1309	Male	7.6	7.5-8.0	35	1-32	534	12	26	—
47 79		13.6	?	150	?	1375	24	—	—
47 79		15.7	8.4	125	1-64	700	18	—	33.0
47 79		15.7	6.2	55	1-16	654	24	—	55.0
1182		11.9	5.87	40	1-16	1060	12	26	—5.9
43 326	Female	13.5	6.02	50	1-32	1039	16	40	4.6
47 184		15.8	5.87	40	1-8	800	9	26	20.0
43 380	Male	13.0	5.55	150	1-128	1360	24	—	35.6

\* Quantitative studies of renal function carried out before during and after the transfusion reaction.

† The aeration method of Van Slyke and Cullen<sup>15</sup> was used to determine the concentrations of blood urea nitrogen.

### EXPERIMENTAL OBSERVATIONS

The renal aspects of hemolytic reactions produced by transfusion of incompatible whole blood were studied in fourteen transfusions given to 8 different dogs. Data of a general character relating to all experiments are summarized in table 1.

The results of single or multiple transfusions were essentially similar with as many as four reactions having been produced in the same animal at varying intervals.

\* Drs. James A. Bush, John W. Harden and Henry Teitelbaum assisted with the radioactive iron activity.

† Determinations of *para* aminohippurate and mannitol clearance were done by Dr. J. H. Cusson and Dr. Christine Watcher.

vals of at least two weeks. The urine at the time of transfusion was alkaline, with a pH of over 7.5, in eight experiments, and acid, pH 5.5 to 6.2, in five experiments. In one instance the pH of the urine was not determined since the reaction occurred unexpectedly after a transfusion of mismatched blood given for another purpose.

Transfusions ranged in size from 35 to 205 ml. of whole blood. When compared with individual dog weights this represented from 2.5 to 15 ml. per kilogram or the approximate equivalent of from 200 to 1000 ml. of blood transfused into a 70 kilogram human being.

The maximum plasma hemoglobin concentration after each transfusion was apparently related both to the amount of blood injected and to the initial antibody titer, the height of which appears to determine to some extent the rapidity of red cell destruction.

Hemoglobin invariably appeared in the bladder urine within five or ten minutes after completion of the transfusion. Exact measurements of the duration of hemoglobinuria were not possible in all experiments, but the shortest period observed was eight hours and none extended beyond twenty-four hours. The variations encountered were unrelated to the pH of the urine, but maximal plasma hemoglobin concentration and body weight were apparently contributing factors. The total amount of hemoglobin excreted by the kidneys ranged from 10 to 40 per cent of that in the transfused blood and came only from this source since in the five experiments involving donor red cells labelled with radio-active iron the isotope content of the total hemoglobin excreted by the recipient's kidneys was identical with that in an equivalent amount of hemoglobin from the donor.

The concentration of urea nitrogen in the blood was determined at daily intervals for periods up to one week after each transfusion. Maximal values obtained are listed in column 9. A transient elevation was noted in most instances usually at 24 hours. This was slightly more marked in the group with acid urine but in all there was a prompt return to the pretransfusion level in from 48 to 72 hours.

Slight proteinuria was noted for a few days after the cessation of hemoglobinuria in some but not all animals with both alkaline and acid urine. There was no consistent alteration in the specific gravity of the urine at any time. Catheterized specimens of urine collected during the period of hemoglobinuria all contained variable amounts of brown granular material while the urinary sediment of dogs with initially acid urine also showed moderate numbers of pigmented casts.

Quantitative studies of renal function were carried out before, during and after the transfusion reaction in six experiments marked with an asterisk in table 1. In each of these a similar, clearly defined pattern was observed with respect to the renal excretion of hemoglobin, the rate of effective renal plasma flow and the glomerular filtration rate. The findings in two characteristic experiments are illustrated in figure 1. It is to be noted that there were no essential differences between the two experiments, in one of which alkaline urine and in the other acid urine was initially being excreted. Plasma hemoglobin concentrations are shown in the top graph and hemoglobin excretion rates are plotted in the second graph. The latter curves are roughly parallel to those of hemoglobinemia down to the

threshold level, and the calculated renal clearances of the pigment are found to be essentially similar to those observed after hemoglobin injection<sup>10</sup>

Minor irregularities in the excretion rates of hemoglobin are related to the transient changes in effective renal plasma flow and glomerular filtration illustrated in the two lowest graphs of figure 1. The biphasic character of these curves

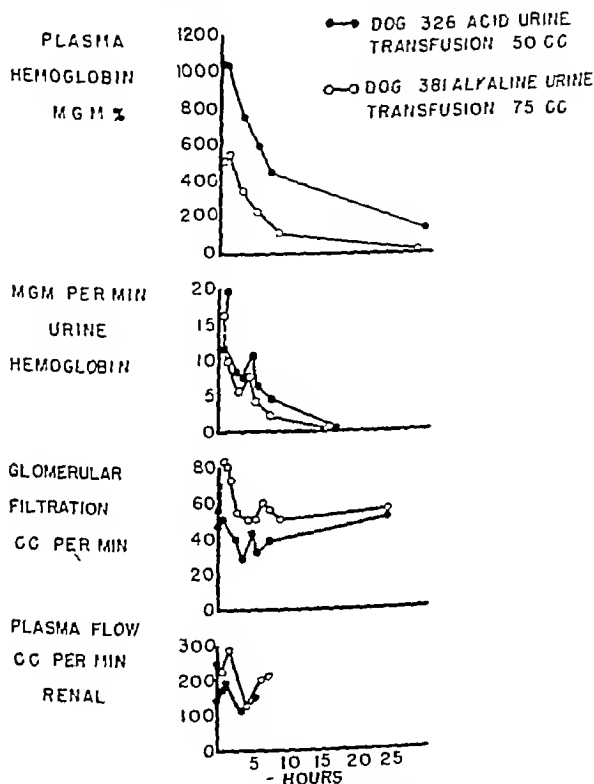


FIG 1—TRANSIENT CHANGES IN RENAL FUNCTION ASSOCIATED WITH HEMOGLOBINEMIA AND HEMOGLOBINURIA PRODUCED BY TRANSFUSION OF INCOMPATIBLE WHOLE BLOOD IN DOGS. Dog 326 Weight 13.5 kilo Antibody titer 1-64 Dog 381 Weight 10.0 kilo Antibody titer 1-16

was a constant finding, with an early rise and a secondary fall below the baseline after several hours. While the degree of these changes was somewhat variable from experiment to experiment, both functions had returned to normal in from six to twenty-four hours in all instances. These transient alterations in renal hemodynamics appear to reflect the general vascular response to a transfusion reaction and indicate that a specific renal vasoconstrictor action of hemoglobin demonstrated some years ago<sup>19, 20</sup> is not an important factor in the development of renal insufficiency.

All animals were well hydrated at the start of each experiment and no oliguria developed, although urine flow was usually reduced for short periods when plasma flow and filtration were at low levels.

Figure 2 illustrates, in two typical experiments, the finding of a temporary alkalinization of the urine during the period of hemoglobinuria which occurred following transfusion of incompatible blood in all animals with initially acid urine. The mechanism of this change is not yet clear but may represent a compensatory effort on the part of the kidney to prevent the accumulation of large amounts of precipitated hemoglobin in the renal tubules. Maximal pH readings coincided with the highest concentrations of hemoglobin in the urine, suggesting that some neutralization results merely from the addition of hemoglobin, which

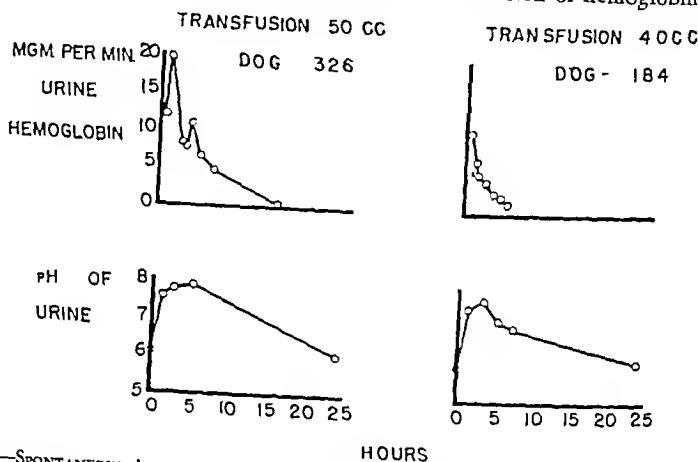


FIG 2—SPONTANEOUS ALKALINIZATION OF URINE DURING PERIOD OF HEMOGLOBINURIA INDUCED BY TRANSFUSION REACTIONS IN DOGS WITH INITIALLY ACID URINE AND NORMAL KIDNEY FUNCTION

has been observed *in vitro* but some interference with the excretion of acid by the lining cells of the distal convoluted tubules is also a possibility.

Two dogs, numbers 1309 and 1182, table 1, were killed twenty-four hours after transfusion reactions, similar to those after which the animals were followed throughout the recovery period. One other animal, 43-326, table 1, having apparently recovered from the transfusion reaction, died suddenly after forty-eight hours. Postmortem examination revealed an acute peritonitis which was attributed to contamination of the *para*-aminohippurate injected intraperitoneally. The kidneys of all three dogs were grossly normal. Histologically, the only finding of note was a small amount of brown, crystalline and granular pigment in occasional distal convoluted and collecting tubules in the two dogs with initially acid urine.

#### DISCUSSION

A critical analysis of the literature dealing with injection of hemoglobin solutions reveals that the mere production of levels of hemoglobinemia comparable

to those seen clinically in most acute hemolytic disorders has little or no damaging effect upon kidney structure or function in normal human subjects or animals

Flink<sup>3</sup> stressed the importance of degree of hemoglobinemia in the development of renal damage, being unable to produce renal injury in dogs unless the initial plasma hemoglobin concentration was 3.7 Gm per 100 ml or the average of the initial and the 24 hour plasma concentrations was 2.2 Gm per 100 ml. From the rather inadequate information available in the literature it is doubtful whether such concentrations ever occur following clinical transfusion reactions and the data presented in this and the preceding paper indicate clearly that the degree of maximal plasma hemoglobin concentration attained is not proportional to the amount of incompatible blood transfused. From the figures in table 1, the plasma concentrations which would have resulted from the sudden liberation of all the hemoglobin in the transfused blood can be calculated. In the experiments in which small transfusions were given the calculated and observed values correspond closely, whereas following larger transfusions the maximal plasma hemoglobin concentrations were only slightly higher than those following small transfusions. This indicates that the degree of hemoglobinemia induced was limited by the ability of the body to destroy incompatible cells. Dog 43-380 illustrates this point, since had all the transfused red blood cells been rapidly hemolyzed, the initial plasma hemoglobin concentration would have been 4.0 Gm per 100 ml instead of the observed concentration of 1.36 Gm per 100 ml.

Although it is obvious from the work of Flink and others that excessive degrees of hemoglobinemia, directly or indirectly, can produce disturbances of renal function, this alone is probably an uncommon cause of hemoglobinuric nephrosis in man. On the other hand there are innumerable clinical and experimental examples of renal insufficiency which have resulted from the association of a moderate grade of hemoglobinemia and some nephrotoxic process.<sup>1, 2, 4</sup> This latter factor can be characterized in some instances as the general or local effect of such agents as shock, ischemia, a chemical poison, infection, or dehydration. However in many acute hemolytic processes, notably those resulting in human subjects from the transfusion of incompatible blood, the cause of serious renal complications is not always clear. Since hemolysis during transfusion reactions is associated with profound changes of a generalized nature, it was considered possible that these might secondarily affect the kidney in a manner comparable to the more specific factors enumerated above. In the present study only normal dogs were used in order to determine whether the combination of these general effects with the concurrent hemoglobinemia was alone sufficient to produce renal insufficiency. Experimental conditions were varied with respect to size of transfusion, antibody titer of recipient, and hydrogen ion concentration of the urine. From the data presented it is apparent that within the range of these variables table 1 only minor, transient changes in renal function were observed. A comparison of the findings in dogs with initially acid urine and those with alkaline urine reveals that in the former, pigment casts occurred in the urine and persisted for 24-48 hours for at least forty-eight hours in the kidneys studied histologically.

that nitrogen retention was slightly more marked. The final outcome, however, was the same in both groups of animals.

Hemolytic transfusion reactions in dogs suffering from shock, anemia, dehydration, and other conditions simulating those for which transfusions are frequently given clinically are being studied at the present time.

### CONCLUSIONS

1. The normal dog's kidney reacts to the transfusion of incompatible dog blood in a manner similar to that observed after hemoglobin injection as far as the excretion of the pigment is concerned.

2. Lowering the pH of the urine to a level of 5.5 has no effect on the final outcome of the reaction nor on the mild, transient alterations in renal function which occur.

3. This type of hemolytic transfusion reaction, similar in most respects to that encountered in human subjects, does not of itself produce renal failure nor the pathological picture of hemoglobinuric or lower nephron nephrosis.

4. The findings in these experiments lend further support to the concept that the development of serious renal complications after a transfusion reaction results from a combination of the hemolytic process with some degree of previous or concomitant kidney damage related to the various clinical states for which transfusion therapy is indicated.

### REFERENCES

- <sup>1</sup> LUCKE B. Lower nephron nephrosis. *Mil Surg* 99: 371, 1946.
- <sup>2</sup> MUIRHEAD E. E., HALEY A. E., HABERMAN S. AND HILL, J. M. Acute renal insufficiency due to incompatible transfusion and other causes with particular emphasis on management. *Blood* 3: Special Issue No. 2 (The Rh Factor) p. 101, 1948.
- <sup>3</sup> ROSS J. F. Hemoglobinemia and the hemoglobinurias. *New Eng. Med. J.* 233: 691, 732, 766, 1945.
- <sup>4</sup> YUILE C. L., GOLD M. A. AND HINDS E. G. Hemoglobin precipitation in renal tubules. A study of its causes and effects. *J. Exp. Med.* 82: 361, 1945.
- <sup>5</sup> FLINK, E. B. Blood transfusion studies. III. The relationship of hemoglobinemia and of the pH of the urine to renal damage produced by injection of hemoglobin solutions into dogs. *J. Lab. & Clin. Med.* 32: 123, 1947.
- <sup>6</sup> YOUNG L. E., ERVIN D. M. AND YUILE, C. L. Hemolytic reactions produced in dogs by transfusion of incompatible dog blood and plasma. I. Serologic and hematologic aspects. *Blood* this issue p. 1218.
- <sup>7</sup> BING R. J. Effect of hemoglobin and related pigments on renal functions of normal and acidotic dogs. *Bull. Johns Hopkins Hosp.* 74: 161, 1944.
- <sup>8</sup> CORCORAN A. C. AND PADE I. H. Genesis of crush syndrome. *J. Lab. & Clin. Med.* 30: 351, 1945.
- <sup>9</sup> DE NAVASQUEZ, S. J. The excretion of haemoglobin with special reference to the transfusion kidney. *J. Path. & Bact.* 51: 413, 1940.
- <sup>10</sup> MONKE, J. V. AND YUILE, C. L. The renal clearance of hemoglobin in the dog. *J. Exp. Med.* 72: 149, 1940.
- <sup>11</sup> (a) LALICH J. J. The influence of injections of homologous hemoglobin on the kidneys of normal and dehydrated animals. *J. Exp. Med.* 86: 153, 1947.  
(b) — The influence of available fluid on the production of experimental hemoglobinuric nephrosis in rabbits. *J. Exp. Med.* 87: 157, 1948.
- <sup>12</sup> DE GOWIN E. L., OSTERHAUGEN H. F. AND ANDERSON M. Renal insufficiency from blood transfusion relation to urine acidity. *Arch. Int. Med.* 59: 432, 1937.

- <sup>11</sup> EVELYN K. A. AND MALLOY H. T. Microdetermination of oxyhemoglobin, methemoglobin and sulfhemoglobin in a single sample of blood J Biol Chem 126 655 1938
- <sup>12</sup> HAHN P. F. Radioactive iron procedures Indust & Engin Chem 17 45 1945
- <sup>13</sup> CHASSIN H. REDISH J., GOLDRING W. RANDES A. J., AND SMITH H. W. The use of sodium p-aminohippurate for the functional evaluation of the human kidney J Clin Investigation 24 583 1945
- <sup>14</sup> SMITH W. W. FINKELSTEIN N. AND SMITH H. W. Renal excretion of hexitols and their derivatives and of endogenous creatinine like chromogen in dog and man J Biol Chem 135 231 1940
- <sup>15</sup> PETERS J. H. The determination of creatinine and creatine in blood and urine with the photoelectric colorimeter J Biol Chem 146 179 1942
- <sup>16</sup> PETERS J. P., AND VAN SLYKE D. D. Quantitative Clinical Chemistry vol 2, Methods Baltimore, The Williams & Wilkins Co 1932
- <sup>17</sup> HESSE, E. AND FILATOV A. Experimentelle Untersuchungen über das Wesen des hämolytischen Schocks bei der Bluttransfusion und die therapeutische Beeinflussung desselben die Nierenfunktionsstörungen im akuten Experiment Ztschr f d ges exper Med 86 211 1933
- <sup>18</sup> MASON, J. B. AND MANN F. C. The effect of hemoglobin on volume of the kidney Am J Physiol 181, 1932.



# EFFECTS OF THE INTRAMUSCULAR ADMINISTRATION OF BAL (2,3 DIMERCAPTOPROPANOL) IN A SUBJECT WITH THE SICKLE CELL TRAIT CASE REPORT

B<sub>3</sub> WILLIAM J KUHN, M D

A VARIETY of studies have indicated that the sickling phenomenon can be accelerated by a number of substances, when any one of the latter is mixed with the appropriate blood, these include carbon dioxide,<sup>1</sup> bacterial cultures, sulfhydryl compounds such as H<sub>2</sub>S, BAL (2,3 dimercaptopropanol), cysteine and glutathione,<sup>2</sup> sodium bisulfite and cevitamic acid.<sup>4</sup> However, there is no clear evidence that individuals harboring the sickle cell trait have ever developed sickle cell anemia, either spontaneously, or when exposed to agents which are known to accelerate sickling in vitro.

The sickle cell trait occurs in about 8 per cent of all Negroes in this country and is generally considered to be distinct from sickle cell anemia. The latter occurs in a much smaller proportion of the Negro population. The former condition is recognizable by observing the development of sickling in moist sealed preparations of freshly drawn blood. Sickle cell anemia is, in addition, associated with a variety of clinical manifestations: anemia, signs of increased blood destruction, abdominal pain, and other diverse effects, most of them related to an increased tendency to circulatory stasis and thrombosis.

The case to be described is one in which acceleration of sickling occurred in an individual harboring the sickle cell trait who was given BAL in oil intramuscularly. Exposure to this known accelerating compound failed to precipitate the picture of sickle cell anemia.

## CASE REPORT

The patient was a Negro female who entered another hospital on July 9, 1948, with complaints of sore throat, fever, headache and rash. The serologic test for syphilis was found to be strongly positive and she was therefore given penicillin and arsenic treatment for six days. Following this she was transferred to the Salt Lake General Hospital where intensive antiluetic treatment with penicillin, mapharsen and bismuth was initiated. On the seventh day of treatment she became disoriented and generally uncooperative. Because she was thought to have developed an arsenical encephalopathy with psychosis, arsenic was discontinued and the patient was given BAL (2,3 dimercaptopropanol) 100 mg in oil intramuscularly every four hours. At this time her temperature ranged from 102 to 103 F. The volume of packed red cells was 51 ml per 100 ml, reticulocytes were 0.5 per cent, the van den Bergh 1:2 mg per cent (indirect 1:1) and the sedimentation rate 3.

In the ensuing ten days she received a total of almost 5 grams of BAL. Shortly after BAL was discontinued the volume of packed red cells was found to be 34 ml and the reticulocyte count was normal. Thrombophlebitis developed at this time and in addition a consolidative process appeared in the lower part of the left lung which was compatible with a pulmonary infarct. The superficial femoral veins were ligated bilaterally. Her mental status remained poor. Serial lumbar punctures showed increases in spinal fluid protein up to 70 mg per cent. The plasma iron was 48 micrograms per 100 ml.

From the Department of Medicine, University of Utah, College of Medicine and the Salt Lake County General Hospital, Salt Lake City, Utah.

Ten days after the administration of BAL the reticulocyte count had increased to 10.4 per cent, the sedimentation rate was 35 ml, and the volume of packed red cells 23 ml. Repeated van den Bergh tests and urine indices remained the same as on the original examination. Clinically the patient showed no evidence of jaundice. White blood counts showed increases up to 20,000 and a shift to the left with a slight myelocytic and myeloblastic response. The differential smear showed red cells which were slightly macrocytic and myeloblastic response. The differential smear showed red cells which were slightly macrocytic and the blood indices were confirmatory. An ascending urinary tract infection developed which was associated with numerous leukocytes in the urine, elevated temperature and BUN, and cultures which were positive for coliform organisms. This was treated successfully with sulfadiazine over the next four weeks. Her impaired mental status persisted as did her anemia. The pulmonary process and thrombophlebitis showed gradual improvement. Reticulocyte counts increased to as high as 13 per cent following which they returned to normal. Urine and fecal urobilinogens yielded consistently normal values. Several previous twenty-four hour sickling preparations had all been negative. However it was later found that sickling preparations which stood for more than twenty-four hours yielded positive findings and it may be assumed that these would have been positive earlier had sufficient time been allowed. In view of the fact that sulphydryl compounds are known to accelerate sickling *in vitro*,<sup>2</sup> it was thought that the use of BAL in this patient may have produced an acceleration of sickling *in vivo* with subsequent thrombotic phenomena and anemia.

TABLE 1—Influence of BAL on Rate of Sickling

Date of Specimen	Percentage of sickled cell Number of hours following withdrawal of specimen						
	0	1	4	8	16	24	48
9/5/48 (prior to second course of BAL therapy)	0	0	0	0	0	0	5
10/4/48			Intramuscular BAL started				
10/7/48 (one hour following injection of 100 mg BAL in oil 1 ml)	0	1*		25*	90		

\* These examinations revealed abnormal type of rouleaux.

In order to ascertain the exact role played by this compound in the genesis of her anemia, it was decided to reinstitute BAL therapy in doses similar to those employed previously. Prior *in vitro* studies were performed with the patient's blood utilizing saturated aqueous BAL. These are described in detail in a subsequent section (see Observations). In brief they indicated that BAL increased the rate of sickling and the viscosity of the blood as judged by fresh BAL treated moist preparations and comparative sedimentation rates.

During the ten day period of BAL therapy there was no appreciable alteration in the volume of packed red blood cells which remained about 35 ml per 100 ml, nor was there any evidence of increased blood destruction. Consistently normal values were obtained for urine and fecal urobilinogen, reticulocyte count and serum bilirubin. It is of interest that in spite of this the rate of sickling was influenced considerably by the administration of BAL (see table 1). The rate of sickling was markedly accelerated one hour after the administration of BAL as compared with that in specimens of blood just prior to the injection of this drug. Corresponding with this it was observed that the erythrocyte sedimentation rate was markedly decreased after the administration of BAL, thus confirming *in vitro* results. The patient showed no evidence of thrombotic phenomena during the trial period with BAL. Her subsequent course has been good with the exception of her mental status which has remained somewhat clouded. Her blood picture has shown steady improvement and all hemolytic indices remained normal. The patient was recently discharged in good condition and with normal blood values.

#### OBSERVATIONS

Routine fresh sealed preparations indicated that the patient's red blood cells sickled slowly. No sickle cells were seen even after twenty-four hours. A third

six hours there was two per cent sickling and at seventy-two hours the majority of the red blood cells were sickled. The use of  $\text{CO}_2$  or  $\text{H}_2\text{S}$  gas bubbled directly through the blood accelerated the rate of sickling considerably, so that small numbers of red cells became sickled immediately and most were sickled at twenty-four hours. Saturated aqueous BAL acted similarly when one drop was mixed with an equal amount of the patient's blood and a fresh sealed preparation was made. It is interesting that following contact with sulfhydryl compounds many of the

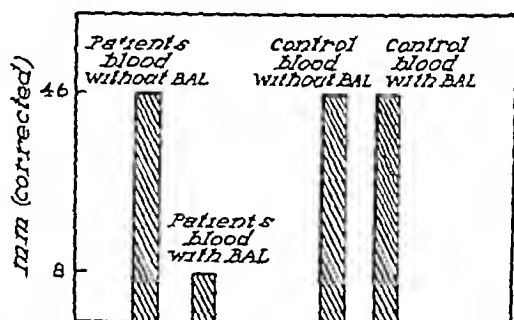


FIG. 1.—THE IN VITRO EFFECT OF BAL (AQUEOUS) ON SEDIMENTATION RATE. (BAL-blood mixtures were prepared by adding one drop of saturated aqueous BAL to 5 cc of whole blood.)

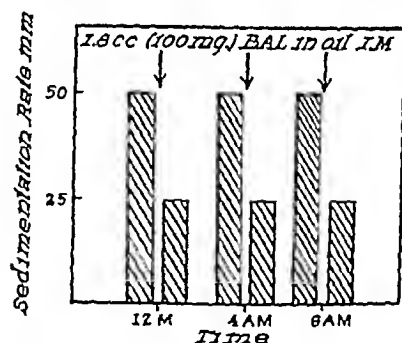


FIG. 2.—EFFECT OF INTRAMUSCULAR INJECTION OF BAL ON SEDIMENTATION RATE IN SUBJECT WITH SICKLE CELL TRAIT

red blood cells appeared more limp and flabby, and rouleaux were of an abnormal type.

Comparative sedimentation rates carried out on the patient's blood with and without the addition of water soluble BAL indicated that the sedimentation rate of BAL treated blood was decreased more than five fold in comparison to untreated blood (see fig. 1). The mixture of BAL with the blood of normal persons, on the other hand, failed to alter the sedimentation rate. BAL-blood mixtures were prepared by adding one drop of saturated aqueous BAL to 5 ml of whole blood. Following institution of the second series of BAL injections, sealed preparations

were made one hour after administration of the drug. These were found to sickle considerably more rapidly than did the preparations made before the drug was given (see table 1). Serial sedimentation rates were found to increase and decrease alternately before and after an intramuscular injection of 100 mgm. of BAL in oil (see fig. 2).

### DISCUSSION

Study of the present case offers several points of interest. In the first place, it emphasizes the necessity for observations of fresh blood preparations over a period of several days when sickling is being sought. Routine 24 hour specimens showed no sickling in the present case whereas specimens observed at later intervals were definitely positive. This, of course, becomes of less importance as knowledge regarding the known accelerating substances accumulates. In the present case, for instance, it was possible to reduce the time of sickling materially by the use of sulfhydryl compounds. This is in conformity with the findings of Thomas and Stetson.<sup>2</sup>

It has been demonstrated, furthermore, that the intramuscular administration of BAL accelerates the rate of sickling. Winsor and Burch<sup>1</sup> obtained comparable effects when they utilized methods which increased the CO<sub>2</sub> concentration in patients with sickle cell disease.

The addition of aqueous BAL to specimens of the patient's blood resulted in retardation of the sedimentation rate, similar results were obtained following the intramuscular injection of BAL in oil. The value of the sedimentation rate in detecting sickling was first recognized by Winsor and Burch,<sup>1</sup> who found that the erythrocyte sedimentation rate of the blood of patients with sickle cell anemia could be slowed or accelerated by alternate saturation with carbon dioxide and oxygen. Inhalation of pure oxygen was found to accelerate the sedimentation rate, on the other hand, keeping a tourniquet on the arm for ten minutes retarded the sedimentation rate, as did also rebreathing into a paper bag. Normal blood was found to be affected only slightly by these gases.

The experiments of Thomas and Stetson have indicated that sulfhydryl compounds similarly retarded the sedimentation rate in individuals with sickle cell disease. Of special interest in the present case was the alteration in sedimentation rate following exposure of the patient to BAL. This occurred, however, in the absence of any of the pathognomonic criteria of sickle cell anemia.

The possible development of sickle cell disease in Negroes harboring the sickle cell trait is a question which has evoked some divergence of opinion. The trait is said by Bauer<sup>3</sup> to change occasionally to the disease under certain conditions of anoxemia and stress if a large number of red blood cells are caused to sickle. Such conditions would include local or general anoxemia resulting from infectious disease, surgical procedures, or other conditions known to slow the circulation of the blood, such as pregnancy and blood transfusion. On the other hand, Wintrobe<sup>4</sup> and Singer, et al.<sup>7</sup> insist that sickle cell trait and sickle cell disease are separate entities, and that carriers of the trait never acquire sickle cell disease. Singer and associates<sup>7</sup> studied the comparative survival rates in normal individuals of trans

fused cells from persons with the trait and with sickle cell anemia and found that the former survived as long as did normal red blood cells, whereas the latter survived a much shorter period of time. On the basis of these studies, they have suggested that sickle cell anemia develops because of an alteration in the red blood cell cytoskeleton which is qualitatively different from the structural anomaly responsible for the sickling phenomenon.

The etiology of the anemia in the present case was not definitely ascertained. Administration of BAL to the patient did not produce any evidence of hemolysis or any of the other characteristics of sickle cell disease. What role, if any, the compound played in relation to the anemic and thrombotic episodes earlier in her clinical course is difficult to determine. It is quite probable that administration of the drug was in no way involved. The manner in which her anemia developed, i.e., without any evidence of hemolysis and concomitant with thrombophlebitis and a consolidative pulmonary process, followed shortly thereafter by a severe renal infection, all suggest an anemia of infection rather than anemia due to blood loss, which is the only other possibility which comes to mind. The low plasma iron might have been found in either case but the absence of hypochromia and microcytosis of the red blood cells, and the lack of any clinical evidence or history of blood loss makes the former possibility the more probable.

#### SUMMARY

The effects of the intramuscular administration of BAL in a Negro harboring the sickle cell trait have been presented. It was observed that the rate of sickling was accelerated and the erythrocyte sedimentation rate was retarded in the presence of BAL both in vitro and apparently in vivo. However, the administration of BAL produced none of the pathologic sequelae characteristic of sickle cell disease.

#### ACKNOWLEDGMENT

I wish to thank Dr. M. M. Wintrobe, Professor and Head of the Department of Medicine, University of Utah College of Medicine for his numerous invaluable suggestions and criticisms.

#### REFERENCES

- <sup>1</sup> WINSOR, T. AND BURCH, G. E. Rate of sedimentation of erythrocytes in sickle cell anemia. *Arch. Int. Med.* 73: 41-52, 1944.
- <sup>2</sup> SINGER, K. AND ROBIN, S. Rapid test for the demonstration of sickle cells and its clinical significance. *J. A. M. A.* 136: 1021-1025, 1948.
- <sup>3</sup> THOMAS, L. AND STETSON, C. Sulfhydryl compounds and the sickling phenomenon. *Bull. Johns Hopkins Hosp.* 83: 176-180, 1948.
- <sup>4</sup> DALAND, G. A. AND CASTLE, W. B. A simple and rapid method for demonstrating sickling of the red blood cells. The use of reducing agents. *J. Lab. & Clin. Med.* 33: 1082-1088, 1948.
- <sup>5</sup> BAUER, J. Sickle cell disease. *Acta med. Scandinav.* 79: 1-11, 1947.
- <sup>6</sup> WINTROBE, M. M. Personal communication.
- <sup>7</sup> SINGER, K., ROBIN, S., KING, J. AND JEFFERSON, R. The life span of the sickle cell and the pathogenesis of sickle cell anemia. *J. Lab. & Clin. Med.* 33: 975-984, 1948.

## RH ISOSENSITIZATION IN THE AMERICAN NEGRO

By MILTON S. SACKS, M.D., JOSHUA A. COHEN, M.D., and J. F. BERRY, M.D., of the Baltimore Rh Laboratory

THE INCIDENCE of Rh isosensitization in the American Negro has been studied by numerous investigators. However, no comprehensive studies on the occurrence of Rh isosensitization have been published. As a result of the paucity of such data, there has been a more or less general impression that Rh isosensitization is less prevalent in the American Negro than in the white population. The purpose of this paper is to report on the basis of a statistically significant series of Negro patients treated in this laboratory.

The material for this study was taken from the files of the Baltimore Rh Laboratory for the period beginning August 1, 1954, and ending November 30, 1955. During this period a total of 55,561 individuals have been studied. The majority of Rh negative individuals are routinely examined for antibodies by testing a small pooled, O MN Rh positive erythrocytes suspended in 30 per cent bovine albumin solution. The specificity of any antibody found is likewise determined. The plan of prenatal study as carried out in this laboratory has been previously published.

### RESULTS OF STUDY

In the group of 55,561 patients there were 43 (15 Caucasoid individuals of whom 8,889 (17.48 per cent) were Rh<sub>0</sub> negative. The remainder, 11,196 were American Negroes of whom 1,302 (8.37 per cent) are Rh<sub>0</sub> negative. The incidence of Rh negative individuals, particularly in the Caucasoid group, is somewhat higher than previously established figures since many such patients, when found to be Rh negative elsewhere, have been referred to us for study. Excluding this factor, the patients constituting this study represent a completely unselected and random group. In the group of 8,889 Rh<sub>0</sub> negative Caucasoid individuals 503 instances of Rh isosensitization were encountered. Among these cases were 34 patients who have been studied through two pregnancies and 9 instances of sensitization by transfusion in males. Correction for these factors leaves a total of 460 isosensitized Caucasoid females. The incidence of isosensitization is, therefore, 5.2 per cent.

Among the 1,302 Rh<sub>0</sub> negative Negro patients 77 instances of Rh isosensitization were encountered. Thirteen of these patients have also been observed through two pregnancies. Thus, the corrected figure of 64 sensitized individuals represents an incidence of isosensitization in the Negro group of 4.9 per cent. The difference in the incidence of isosensitization between the Caucasoid and Negro group was found to be insignificant on statistical analysis.\*

From the Baltimore Rh Laboratory and the Departments of Medicine and Obstetrics, the University of Maryland School of Medicine, Baltimore, Md.

\* The formula for the standard error ( $\sigma$ ) of the difference between 2 percentages is

## CLINICAL ASPECTS

Examination of the clinical records of this group of patients demonstrated a significant fact which may be one of the reasons for the existing general impression concerning the rarity of erythroblastosis fetalis among the American Negroes. In contrast to the Caucasoid group, approximately one half of the infants were inadequately studied during the neonatal period. These patients were hospitalized in many different institutions throughout the city. Thus many relatively mild cases of erythroblastosis fetalis were undoubtedly over-looked. In spite of this fact, 14 cases of clinically obvious hemolytic disease of the newborn were encountered in the series. Of the latter group 4 were severe enough to lead to death. As will be observed in table 1 the degree of isosensitization observed in 64 cases is entirely comparable to that seen in Caucasoid individuals. Yet, the immediate mortality rate of 6.2 per cent is distinctly lower than the overall mortality from congenital hemolytic disease. Whether this is actually representative of the true situation, or whether this is simply another evidence of inadequate clinical follow-up, cannot be accurately stated at this time.

Two illustrative examples of erythroblastosis fetalis in the American Negro will be cited.

*Case 1.* H. S., a 43-year-old Negro, para 7 with 6 living children, was first seen in the last trimester of her pregnancy. The past history was negative for previous blood transfusions and erythroblastosis fetalis. She was found to belong to type O MN Rh negative. Serologic studies revealed a nonvalent antibody in titer of 3 units with serum-suspended cells, and 6 units with albumin-suspended cells. The blocking test was negative. Antibody specificity was Rh<sub>0</sub>. Serologic studies were carried out every two weeks, and a significant rise in titer was observed. On her last visit, two weeks before delivery, the antibody titer with serum-suspended cells was found to be 48 units while the titer with albumin-suspended cells was 384 units. The blocking test was now strongly positive. She delivered an infant weighing

$$\sigma_D = \sqrt{\frac{p^1 q^1}{n_1} + \frac{p^2 q^2}{n}}$$

where

p = per cent of nonsensitized cases  
q = per cent of sensitized cases

and

n = total number of cases

$$\sigma_D = \sqrt{\frac{0.94825 \times 0.5175}{8,889} + \frac{0.95084 \times 0.04916}{1,302}}$$

$$\sigma_D = 0.65\%$$

$$\text{Difference (D)} = 5.175\% - 4.916\% = 0.26\%$$

$$\frac{\sigma}{\sigma_D} = \frac{0.26}{0.65} = 0.4$$

so that difference is not statistically significant

3335 grams whose blood type was O MN Rh. The initial hemoglobin level was 15 grams and there were 22 per cent nucleated red blood cells in the peripheral blood. Jaundice appeared two hours after birth and lasted three days. Hepatomegaly and splenomegaly were also present. The infant received no transfusions and was discharged from the hospital apparently clinically normal on the fifth postpartum day. On the twentieth postpartum day examination in the Pediatric Clinic revealed the liver and spleen to be barely palpable. There was no jaundice but blood study revealed a hemoglobin of 4.0 grams. Multiple transfusions of fresh type O MN Rh negative blood were given with subsequent uneventful recovery.

*Case 2.* S. A. 20-year-old Negro para 2, with 2 living children was first seen in the thirty-second week of her pregnancy. The past history revealed no instance of previous blood transfusions nor of any previous erythroblastic infants. The patient's blood type was found to be O MN rh, that of her husband O MN Rh, Rh<sub>1</sub> (probable genotype R<sup>1</sup> R<sup>1</sup>). Both previous children were O MN Rh, rh (genotype R<sup>1</sup> r). Initial serologic studies revealed univalent antibodies in a titer of 96 units with albumin suspended cells. The blocking test was negative. Antibody specificity was Rh<sub>0</sub>. Blood studies at biweekly intervals demonstrated practically no rise in the antibody titer. On the day prior to delivery serologic study revealed a titer of 196 units with albumin-suspended cells and only 2 units with serum suspended cells. There were no agglutinins active in saline solution and the blocking test was negative. The patient was delivered of an infant weighing 3285 grams whose blood type was O MN Rh<sub>1</sub>. The initial hemoglobin

TABLE 1—Antibody Titers in Various Cell Suspension Media in 64 Isosensitized Negro Women

Units of antibody	Suspension media			Blocking test
	Physiologic saline solution	Pooled human serum	30% bovine albumin solution	
0	42 cases	10	0	Positive 30 cases
1-10	28 cases	37	9	Negative 34 cases
10-100	4 cases	17	24	
100-1000	0 cases	0	15	
1,000-10,000	0 cases	0	6	

\* Ten cases not studied with this medium

was 15 grams and blood smears showed 4 per cent nucleated red cells. Because of a falling hemoglobin a 90 cc. transfusion of fresh O MN Rh negative blood was given to the infant on the second day of life. Severe jaundice was observed on the second day associated with hepato-splenomegaly. Despite transfusions every other day the hemoglobin continued to drop. During the next forty-six days numerous small transfusions were necessary to maintain a satisfactory hemoglobin level. In all a total of 620 cc. of blood was given over the forty-six day period.

These cases, which are presented to illustrate the severity of erythroblastosis fetalis in Negro infants, by no means illustrate ideal methods of management. It is rather interesting to observe that Case 1 illustrates a variety of erythroblastosis fetalis not infrequently encountered in which the development of marked anemia occurred three to four weeks after delivery in an infant apparently clinically normal, during the early neo-natal period. In view of the current procedure of early discharge of postpartum patients, this variety of erythroblastosis fetalis is undoubtedly overlooked unless special attention is paid to blood studies during the first four to six weeks of life.

Since the incidence of the Rh-negative type in the American Negro (8.4 per cent) is only somewhat more than one half of that in Caucasians (13 to 15 per cent) the actual number of cases of erythroblastosis fetalis in Negroes will be correspondingly lower. Nevertheless, survey of a large series has revealed that there is no significant



difference in the incidence of isosensitization of Rh<sub>0</sub>-negative individuals in either group Rh isosensitization and erythroblastosis fetalis may be expected to occur in the American Negro in direct proportion to the incidence of the Rh-negative type in that race. Similar observations have been made in other races.<sup>3</sup>

#### SUMMARY

1. Studies of the Rh factor in 11,486 pregnant female American Negroes revealed 1,302 who were Rh<sub>0</sub> negative (8.4 per cent). Sixty-four cases of isosensitization were encountered which gave an incidence of 4.9 per cent in the Rh-negative patients. In comparison, among 8,889 Rh<sub>0</sub>-negative Caucasians, 460 cases of isosensitization (5.2 per cent) were encountered. The difference was found to be statistically insignificant.

2. Two typical examples of erythroblastosis fetalis in American Negro infants are presented.

#### REFERENCES

- <sup>1</sup> WIENER, A. S. AND WEXLER, I. B. Erythroblastosis in Negroid infants. *Blood* 7: 414, 1948.
- SACKS, M. S., KLUENTZ, W. J., AND JAHN, E. F. Studies in Rh isoimmunization in pregnancy. *Am. J. Obst. & Gynec.* 54: 400, 1947.
- LEVINE, P. On human anti Rh sera and their importance in racial studies. *Science* 96: 145-1942.

# THE PATHOGENESIS OF ERYTHROBLASTOSIS FETALIS

By B S KLINE, M D

**M**ICROSCOPIC examination of numerous sections of the placenta of an 8½ month pregnancy in a case of erythroblastosis fetalis, in which the baby lived 33 minutes, and of the placenta of a 7½ month pregnancy in a similar case in which the baby lived 25 minutes, showed occlusion of peripheral blood vessels of many villi and trunks by agglutinated red blood cells and fibrin. Associated with the vascular thromboses, there were, in places, necrosis of the walls and of regional tissues with rupture and hemorrhage of fetal blood, containing numerous intact nucleated red blood cells, into regional intervillous spaces. Through the broken surfaces, adjacent maternal blood was in contact with the fetal circulation.

These observations indicate the mechanism of transfer to the mother of incomparable fetal red blood cells in cases of erythroblastosis fetalis and of the transfer to the fetus of the maternal antibody that produces the anemia.

The two placentas showed, in addition to hemorrhages that apparently occurred at or very shortly before the time of expulsion, others somewhat older with abundant fibrin and red blood cells, some with degenerating nuclei, covering the ruptured surfaces of villi and trunks, indicating that the intermingling of fetal and maternal bloods had been stopped by the clotting of fetal blood at the sites of hemorrhage.

Vascular thromboses with necrosis and rupture of peripheral tissues of many villi and trunks and hemorrhage of fetal blood into regional intervillous spaces was observed in the placenta of all 13 additional cases of erythroblastosis fetalis and of all 213 cases in the last half of normal pregnancy examined and reported previously.<sup>1</sup> Although the changes in the 213 placentas of the last half of normal pregnancy observed microscopically are the same as those in the two cases of erythroblastosis fetalis described above, it is doubtful if the fetal hemorrhages into intervillous spaces would have been recognized as such, without the previous identification of unquestionable fetal hemorrhages with nucleated red blood cells into intervillous spaces in the two placentas here reported.

Since the first report, by Levine and Stetson<sup>2</sup> of transplacental transfer of an immunizing blood factor inherited from the father various explanations of the mechanism concerned have been offered.

The permeability of the placenta of mammals has been found to increase progressively to the end of pregnancy as the layers of tissue between maternal sinuses and fetal circulation diminish (Flexner and Gellhorn<sup>3</sup>). Levine<sup>4</sup> has assumed that the thinning of the placental barrier and the pressure in the fetal circulation, greater than in the local maternal sinuses, afford ample opportunity for the escape into the sinuses of a minute number of fetal red blood cells in one or another form.

Haldane<sup>5</sup> is of the opinion that the abnormal permeability of the placenta

From the Laboratory Department Mt. Sinai Hospital Cleveland, Ohio

to the passage of an antigen from the fetus to the mother is at least often genetically determined. Burnham<sup>6</sup> has suggested that subclinical deficiency of vitamin C in the mother might be sufficient to permit a break in the integrity of the capillaries of the chorion with escape of Rh positive fetal blood, thus leading to isoimmunization of the Rh negative mother. Naeslund and Aren<sup>7</sup> have recently reported a case of toxemia of pregnancy in which the full term placenta showed multiple gross hematomas and microscopically in these areas, gaps in the epithelium of villi and in the walls of their blood vessels. The photomicrographs show



FIG. 1.—Placenta #1979 of 8½ month pregnancy. Case of erythroblastosis fetalis (Magnification about 300 X) (Right middle area) Occlusion of blood vessel of villus by agglutinated red blood cells and fibrin. Necrosis of walls and of regional tissues with rupture.

marked vascular engorgement suggesting that the regional fetal hemorrhage was due to rupture following obstruction to the return of venous blood at or very shortly before the expulsion of the placenta.

Javert<sup>8</sup> has reported the finding of gross hematomas in the placenta of 8 of 34 cases of erythroblastosis neonatorum and in 7 of 10 examined microscopically, nucleated fetal red blood cells were found.

The observations especially in the two cases reported here and also in all the additional 13 cases of erythroblastosis fetalis and in all the 213 cases in the last half of normal pregnancy studied, indicate that hemorrhage of fetal blood from many villi and trunks into the regional intervillous spaces occurs in the last half of all pregnancies as a result of occlusion of the involved peripheral blood



FIG 2.—Placenta #2243 of 7½ month pregnancy Case of erythroblastosis fetalis (Magnification about 150 X ) (Middle lower area ) Very recent hemorrhage from villus into regional intervillous spaces (Many of the fetal red blood cells are nucleated )



FIG 3.—Placenta #2243 (Magnification about 200 X ) (Center) Recent hemorrhage from villus into regional intervillous spaces showing early formation and molding of clots (Some of the fetal red blood cells are nucleated )



FIG 4—Placenta #1979 (Magnification about  $300\times$ ) (Left and upper) Recent clot containing numerous intact and degenerating nucleated fetal red blood cells (especially upper right) following hemorrhage from villus (Reprinted by permission of the American Journal of Obstetrics and Gynecology)



FIG 5—Placenta #1979 (Magnification about  $350\times$ ) (Center and right.) Clot containing intact and degenerating nucleated fetal red blood cells following hemorrhage from villus



FIG 6—Placenta #1979 (Magnification about  $200\times$ ) (Left lower and center right upper) Fetal hemorrhage from peripheral vessels of trunk with recent blood clot formation



FIG 7—Placenta #2330 of a 9 month pregnancy Case of erythroblastosis foetalis (Magnification about  $400\times$ ) Villi with double epithelial lining and thick stroma

vessels by agglutinated red blood cells and fibrin and necrosis and rupture of the walls and of the regional fetal tissues<sup>1</sup> It is possible that the primary damage is due to the excretion of waste products of metabolism

The placenta in every one of the 228 cases reported showed, in addition to recent fetal hemorrhages, older ones with the clots composed mostly of fibrin stained by hemoglobin of laked fetal red blood cells, and degenerating and degenerated fetal red blood cells Fetal blood clots, apparently still older, consisting of little more than fibrin are the most conspicuous finding in all placentas after mid-pregnancy The oldest clots were observed in various stages of organization

In the placenta of 8 of the 15 cases of erythroblastosis fetalis, the villi showed the changes characteristic of the disease They were much thicker than normal and some were edematous The surface epithelium was thickened and in places double The stroma was thicker than average and compact where not edematous Many of the central vessels of villi and trunks showed degenerative changes and shrinkage, in places complete obliteration Some showed thickened endothelium and prominent perivascular fibrosis

The villi of erythroblastosis fetalis, with thick double epithelial covering and thick stroma, resembled those of the first few months of normal pregnancy The thickening and the doubling of the epithelial covering, the thickening of the stroma and the other changes may well have been a response to their constant exposure to the harmful fetal red blood cell antibody of the maternal blood

Since it is now known that the erythroblastosis is a secondary manifestation, the designation *erythroblastosis fetalis* for the disease, is by no means satisfactory and appears to be no longer justified Furthermore, since it has been found that destruction of the incompatible fetal red blood cells in the disorder may occur in part by phagocytosis (references given in a previous article<sup>1</sup>), the term *hemolytic disease of the newborn* is not entirely accurate A more fundamental designation in keeping with the author's concepts would be "transplacental erythrocytotoxic anemia

#### SUMMARY

The mechanism of transfer, in cases of erythroblastosis fetalis, of incompatible fetal red blood cells to the mother and of maternal blood with antibody to the fetus, was observed especially well in 2 cases in which the infants were born alive

The two placentas showed occlusion of peripheral blood vessels of many villi and trunks by agglutinated red blood cells and fibrin Associated with the vascular thromboses, there were, in places, necrosis of the walls and of regional tissues with rupture and hemorrhage of fetal blood, containing numerous intact nucleated red blood cells, into regional intervillous spaces Through the broken surfaces, adjacent maternal blood was in contact with the fetal circulation

A more accurate designation for erythroblastosis fetalis would be "transplacental erythrocytotoxic anemia

## REFERENCES

- <sup>1</sup> KLINE B S Microscopic observations of the placental barrier in transplacental erythrocytotoxic anemia (erythroblastosis fetalis) and in normal pregnancy *Am J Obst & Gynec* 56 226-237 1948
- <sup>2</sup> LEVINE, PHILIP AND STETSON RUFUS E An unusual case of intra group agglutination *J A M. A.* 113 126 1939
- <sup>3</sup> FLECKNER L. B , AND GELLHORN A The comparative physiology of placental transfer *Am J Obst & Gynec* 43 965 1942
- <sup>4</sup> LEVINE, PHILIP The mechanism of transplacental isoimmunization *Blood* 3 404 1948
- <sup>5</sup> HALDANE J B S Selection against heterozygosis in man *Ann Eugenics* 11 333 1942
- <sup>6</sup> BURNHAM L. Vitamin C deficiency as a possible factor in the pathogenesis of erythroblastosis fetalis *Am J Obst & Gynec.* 44 920 1942.
- <sup>7</sup> NÄRBLUND J AND AREN P Studies in changes in the placenta with particular reference to possible injuries to villi and the foetal vessels *Acta Obst et Gynec* 27 115 1947
- <sup>8</sup> JAVERT, C T Further studies on erythroblastosis neonatorum of obstetric significance *Am J Obst & Gynec* 43 921, 1942.



# CORRELATION BETWEEN THE MEAN CORPUSCULAR VOLUME AND RETICULOCYTOSIS IN PHENYLHYDRAZINE ANEMIA IN SWINE

By F DOUGLAS LAWRASON, LT (JG), MCR, USNR, D C ELTZHOLTZ, CPHM, USN, C R SIPE, CPHM, USN, AND P K SCHORA, CPHM, USN

ONE OF THE causes of an increase in mean corpuscular size is a pronounced reticulocytosis.<sup>10</sup> The increase in the volume of the red blood cells due to this cause is usually a temporary finding which follows a sudden loss of blood, a hemolytic crisis, or any reaction which acutely stimulates the hematopoietic system. During treatment of pernicious anemia with specific therapy, it is not unusual to find in conjunction with the reticulocyte response a transient increase in the degree of macrocytosis as measured by the mean corpuscular volume.

When a macrocytic blood picture is associated with a reticulocytosis, it is often difficult to evaluate to what extent the larger size of the reticulocyte contributes to the mean corpuscular volume. This problem was encountered in the interpretation of hematologic data gathered during previous studies with swine at the Naval Medical Research Institute, Bethesda, Maryland. The present investigation was undertaken to study the correlation between the increased percentage of reticulocytes produced by phenylhydrazine hydrochloride and the mean corpuscular volume in swine under controlled conditions.

Phenylhydrazine has been used by numerous workers to produce both experimental anemia and reticulocytosis.<sup>1-7</sup> This drug and its derivatives have been considered hemolytic agents.<sup>8-9</sup> However, Goodman and Gilman<sup>10</sup> do not consider the chemical action of the drug hemolytic in nature. They believe the drug enters the red cell, splits part of the hemoglobin to hemin and denatured globin, and the hemin, acting as a catalyst, changes the remaining hemoglobin to methemoglobin and possibly other unidentified substances. Phenylhydrazine usually does not cause depression of the bone marrow and probably does not affect the immature red cell<sup>11</sup> or the white cell.<sup>12</sup> Erythroid and myeloid hyperplasia have been noted in bone marrow of animals treated with the drug.<sup>1-12-17</sup>

## MATERIALS AND METHODS

Six adult swine averaging 185 pounds in weight were used for these studies. They were procured from a hybrid stock predominantly Duroc Jersey with an admixture of Poland-China and Chester White. All six swine were kept in a common pen measuring approximately 10 by 20 feet. Their diet as recommended by the U S Department of Agriculture consisted of a 17 per cent protein-vitamin mineral mixture and 83 per cent whole yellow corn. Brucella abortus agglutination tests were negative.

Animals were bled in the fasting state. The blood was obtained from the deeply lying jugular veins in the lower neck. The bone marrow was aspirated from the sternum with a Turkel needle and approximately 0.1 to 0.2 cc. of marrow blood was withdrawn. The preparation of the smear-imprint was made immediately upon aspiration. All other laboratory determinations were carried out by standard methods.

From the Naval Medical Research Institute, National Naval Medical Center Bethesda Maryland  
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The same amount of phenylhydrazine hydrochloride was given to all of the animals on each day of treatment. The drug was given orally with a handful of food for the first nine doses. Beginning with the tenth dose and continuing to the end of the study it was given intravenously in a 2 per cent aqueous solution into the same plexus of veins from which the animal was bled.

### RESULTS

Initially all 6 swine were considered hematologically normal with an average erythrocyte count of 6.9 million per cu. mm. (fig. 1). The average mean corpuscular

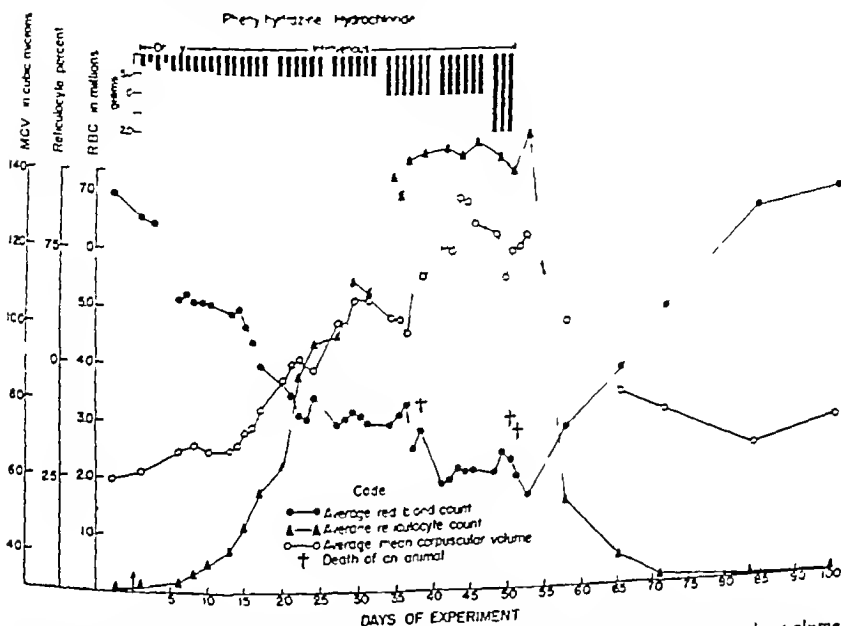


FIG. 1.—Course of the average red blood count, reticulocyte count, and mean corpuscular volume in the five adult swine administered phenylhydrazine hydrochloride. The amount of phenylhydrazine HCl and time of administration is shown at the top of the graph.

volume (MCV) of the red cells for the group was 60 cubic microns and the reticulocyte count was 0.3 per cent. Phenylhydrazine hydrochloride was fed by mouth in daily doses of 0.2 to 0.4 Gm. for the first nine days. The dose was increased to 0.5 Gm. on the tenth day and was given intravenously (fig. 1). Little or no immediate reaction was noted with the administration of 0.5 Gm.; however, when 1.0 Gm. was given intravenously the animals exhibited mild to moderate weakness following injection. This reaction lasted from one to three minutes. On the forty-second dose the drug was increased to 2.0 Gm. and the weakness following the injection became more severe and lasted five to ten minutes. The severe response observed with the higher dose may have been due to the fact that when it was administered, all animals were severely anemic, listless, and weak. On the fortieth day the drug was discontinued.

One animal, number 5, failed to respond with a reticulocytosis. It became rapidly leukopenic and severely anemic and died from intercurrent infection on the fourteenth day, after receiving only 4.4 Gm of phenylhydrazine. At autopsy the bone marrow exhibited an extreme hypoplasia of both erythroid and myeloid components. Since the response of this pig diverged widely from the rest of the group

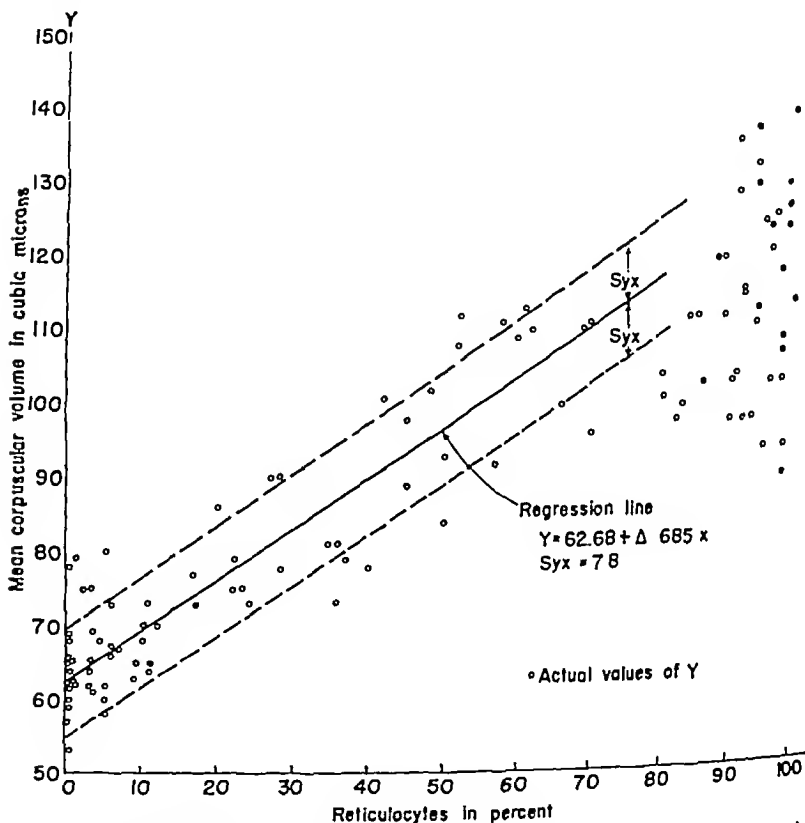


FIG. 2.—Relation of mean corpuscular volume to per cent reticulocytes in five swine treated with phenylhydrazine hydrochloride

and from the usual response to this drug, the data gathered from this animal were not included with the data of the group

The total dose given to the 5 remaining animals ranged from 0.30 to 0.35 Gm per kilogram of body weight, or approximately 28 Gm. Of these 5 pigs, 3 died between the thirty-ninth and fiftieth day and the remaining 2 survived and recovered. The total dose received by each of the 2 surviving animals was 30.7 Gm. One animal was found to be pregnant toward the end of the experiment. Incomplete abortion occurred two days before death of the animal. During the last ten



FIG. 3 —Photomicrographs of bone marrow specimens obtained before and during the administration of phenylhydrazine HCl (a) Bone marrow from one of the swine showing erythroid hyperplasia and multinucleated erythroblasts observed during the period of pronounced reticulocytosis and anemia (b) Bone marrow showing numerous very immature erythroblasts during the period of hyperplasia (c) Erythroid hyperplasia exhibiting many basophilic normoblasts (d) Erythroid hyperplasia exhibiting late normoblasts (e) Normal bone marrow obtained from one of the swine before the administration of phenylhydrazine HCl

days of the study all of the animals developed sterile abscesses at the sites of injection

Figure 1 demonstrates the course of the average red blood count (RBC) for the group of 5 swine. The RBC of 2 of the animals dropped below one million shortly before death but the lowest average level for the entire group was 1.6 million. It will be noted that the average reticulocyte count for the 5 animals was maintained above 50 per cent for one month, and over 90 per cent for sixteen days. During ten days of this latter period, 3 of the animals maintained a reticulocytosis of approximately 100 per cent. The hemoglobin and hematocrit decreased proportionately to the RBC but are not shown on the graph. At the time when the reticulocytosis was marked and when numerous Heinz-Ehrlich bodies were seen, the spectrophotometric determinations of the hemoglobin may have given falsely high readings because of the peculiar turbidity of the solution. A similar turbidity has been described for *in vivo* and *in vitro* studies with phenylhydrazine and has been considered to be due to the release of the Heinz-Ehrlich bodies from the erythrocytes.<sup>11</sup>

The average MCV closely followed the trend of the reticulocytosis and both reached a maximum simultaneously. The maximum MCV of the group average was 133 cubic microns (fig. 1). The MCV of one of the surviving animals which had maintained a reticulocytosis close to 100 per cent for ten days remained at 140 cubic microns during this period. In figure 2 the MCV is plotted with relation to the reticulocyte per cent for all determinations made in the five swine during the study. The regression line for this correlation is a straight line fitted to the data by the method of least squares. In determining this regression line, only the data up to 80 per cent reticulocytes were included. It can be seen that with each increment of 10 per cent in the reticulocytes the MCV increases 6.8 cubic microns plus or minus a standard deviation of 7.8 cubic microns. The reason for using the selected data will be discussed.

The bone marrow of all animals was studied periodically. Beginning at the eleventh to twentieth day and continuing throughout the time of administration of the drug, the bone marrows of the 5 swine exhibited marked erythrocytic hyperplasia (figs. 3a, 3b, 3c, 3d). The myeloid-erythroid ratio was reversed. From 25 to 75 erythroblasts were encountered for every immature white cell. Many of the erythroblasts were quite immature and commonly found in large aggregates containing many pronormoblasts and basophilic normoblasts. Frequent multinucleated erythroblasts were seen (fig. 3a). Mitoses occurred in every stage of maturation and unusual numbers were seen occurring in large nests of erythroid regeneration. A normal pig bone marrow is shown for comparison in figure 3e. The myeloid series did not appear to be disturbed. The peripheral leukocyte counts were erratic throughout the experiment but at no time did they reach leukopenic levels in any of the five animals. Most of the swine developed a leukocytosis terminally which was probably, for the most part, a reaction to terminal infection.

#### DISCUSSION

Clinically, a delayed reaction to phenylhydrazine, manifested by a progressive anemia sometimes occurring many days after discontinuation of the drug, is well

known<sup>18, 19</sup> Experimentally, the effect of the drug upon the red cells seems to occur with little delay. Upon discontinuance of the drug, recovery from the anemia promptly occurs.<sup>20</sup> In the 2 surviving swine no delayed effect of the drug on the RBC was observed.

There is some uncertainty as to whether the action of phenylhydrazine is hemolytic in character<sup>10</sup> or is due to the aplastic effect of the benzol ring as some investigators believe.<sup>9, 16</sup> At any rate, animal number 5 reacted as if it were poisoned with benzol. The pig developed a marked leukopenia and progressive anemia within the first week and died on the fourteenth day of treatment. At autopsy an extensive hypoplasia of all elements in the bone marrow was found. The other 5 swine exhibited the usual response to phenylhydrazine. The variation in response observed in the one animal can not be explained.

The much discussed Heinz-Ehrlich bodies were seen in peripheral blood and for the most part appeared to be within the adult erythrocytes. Cruz<sup>11</sup> considers this fact, among other evidence, to support the theory that phenylhydrazine attacks only the adult and not the immature cell. He believes that these refractile bodies are evidence of destruction within the red cell. No observations were made in this study on whether or not phenylhydrazine attacks only the adult erythrocyte. However, in view of the extreme erythroid hyperplasia in the bone marrow and the high reticulocytosis in the peripheral blood during intravenous administration of the drug, it would appear that the drug did not attack the immature red cell.

The data used for the calculation of the regression line seen in figure 2 were those occurring below the 80 per cent reticulocyte level. Above this level a more extensive scattering of points and apparent lack of continued close linear correlation occurred. During this period of observation the animals were extremely ill, severely anemic, and 3 of the 5 died. The 2 surviving pigs appeared moribund when the drug was discontinued. Wintrobe<sup>20</sup> has pointed out that in pernicious anemia a close correlation exists between the erythrocyte count and the MCV of the red cells when the anemia is moderate, but when the anemia is extreme a close correlation is not found. Similarly, in these swine, the correlation between the number of reticulocytes and the MCV probably was affected by the severity of the anemia.

Qualitatively the bone marrow during this period did not appear to be as hyperplastic as it did earlier in the experiment. Even though the majority of the red cells in the peripheral blood were reticulocytes, it does not seem likely that the erythroid regeneration in the bone marrow could have been proceeding at an ideal maximum rate since the general metabolism was undoubtedly severely disturbed. It may be that at the higher dose of phenylhydrazine practically all of the adult erythrocytes were destroyed thus leaving only reticulocytes in the peripheral blood. Therefore, at the near 100 per cent level the reticulocyte response is probably only an apparent maximum and not a true index of optimum erythrocytic regeneration.

However, in spite of the wide scattering of values between the 80 and 100 per cent reticulocyte level, it appears that the majority of the points tend to cluster toward MCV values lower than expected. The reason for this is not entirely clear. If the animals were iron deficient during this period of observation the smaller mean corpuscular volume of the red cells may possibly be explained. Experiment 1

anemia produced by phenylhydrazine usually is not considered to be complicated by an iron deficiency since the iron from the destroyed cells is returned to the system for new hemoglobin formation. However, sterile abscesses developed at the site of injection of the drug in all of the swine. Robschey-Robbins and Whipple<sup>21</sup> and others<sup>22-23</sup> have shown that in the presence of a chronic inflammatory reaction, such as a sterile abscess, the rate of production of new hemoglobin diminishes because iron is diverted to the tissues and is not made available for hemoglobin synthesis. Therefore, toward the end of the experiment the anemia may have become an iron deficiency anemia.

Another explanation is based upon the previously discussed possibility that the bone marrow was less active during the period when the majority of circulating cells were reticulocytes. Thus, the relative percentage of nearly mature, and therefore smaller, reticulocytes would increase. This trend, if pronounced, may have become sufficient to account in part for the apparent shift of the previously linear correlation. In the final analysis, it is likely that many factors affected the correlation in this high range of reticulocytosis.

Below a reticulocytosis of 80 per cent, a close correlation between the per cent of reticulocytes and the mean corpuscular volume was found. However, since there is a large variation in individual determinations as evidenced by the standard deviation of 7.8 cubic microns, it would be difficult to attribute a macrocytosis to an associated reticulocytosis if the reticulocytes were not increased beyond 10 per cent. Nevertheless, from the data it is possible to determine approximately the role a moderate reticulocytosis would play in the increased mean corpuscular volume found in a macrocytic blood picture in swine.

#### SUMMARY AND CONCLUSIONS

1. Six adult swine were given phenylhydrazine hydrochloride orally and intravenously. Hematologic observations, which included periodic bone marrow studies were made before, during, and after the administration of the drug.

2. Five swine responded to the drug in the usual manner with progressive anemia, reticulocytosis, and erythrocytic hyperplasia of the bone marrow. Three animals died between the thirty-ninth and fiftieth day of the experiment after receiving a total dose of 0.30 to 0.35 Gm. per kilogram of body weight. Two swine survived and recovered after receiving a similar dose.

3. One animal died on the fourteenth day of the experiment and exhibited a course which closely resembled that of benzol poisoning. Rapid and progressive granulocytopenia, anemia, and extreme universal hypoplasia of the bone marrow were observed.

4. A direct correlation between the mean corpuscular volume of the red cell and the per cent reticulocytes was found within the limits of 0 to 80 per cent reticulocytosis. With each increment of 10 per cent in the reticulocytes the mean corpuscular volume increased approximately 6.8 cubic microns.

#### REFERENCES

1. BODANSKY, M., MARR, W. L., AND BRINDLEY, P. An experimental study of the action of phenylhydrazine HCl and acetylphenylhydrazine (Pyrodon) with reference to their use in the treatment of polycythemia vera. *Am J Clin Path.* 2: 391, 1932.

- CARTWRIGHT G E WINTROBE M M AND HUMPHREYS S Studies on anemia in swine due to pyridoxine deficiency together with data on phenylhydrazine anemia J Biol Chem 133 171 1944
- <sup>1</sup> KATCHOURA PAR V Action du chlorhydrate de d-1 acetylphenylhydrazine sur le nombre des globules rouges et des reticulocytes Sang 9 573 1935
- <sup>1</sup> LONG P H Effect of phenylhydrazine derivatives in treatment of polycythemia J Clin Investigation 2 315 1926
- <sup>1</sup> RAPPOPORT S GUEST G M AND WINO M Size hemoglobin content and acid soluble phosphorus of erythrocytes of rabbits with phenylhydrazine induced reticulocytosis Proc Soc Exper Biol & Med 57 344 1944
- <sup>1</sup> SMITH M I AND STOHLMAN E F The influence of vitamin B on hematopoiesis in experimental anemia of the albino rat Pub Health Rep 49 1569 1934
- <sup>1</sup> WATSON C J AND CLARKE W O The occurrence of protoporphyrin in the reticulocytes Proc Soc Exper Biol & Med 36 65 1937
- <sup>1</sup> HOPPE, S G Über die wirkung des phenylhydrazine auf den organismus Ztschr f Physiol Chem 9 34 1884-85
- <sup>1</sup> HUEPPE W C Pathological studies on organic effects of various hydrazine derivatives J Indust Hyg & Toxicol 18 1936
- <sup>1</sup> GOODMAN L AND GILMAN A The Pharmacological Basis of Therapeutics New York The Macmillan Co 1941 P 1150
- <sup>1</sup> CRUZ W O Acetylphenylhydrazine anemia I The mechanism of erythrocyte destruction and regeneration Am J M. Sc 202 781 1941
- <sup>1</sup> McNAMARA D H AND SANBURN W D Phenylhydrazine poisoning JAMA 96 268 1931
- <sup>1</sup> LONG P H Experimental anemia produced by phenylhydrazine derivatives J Clin Investigation 2 329 1926
- <sup>1</sup> ALLEN E V AND GIFFIN H Z Experiments with phenylhydrazine II Studies on renal and hepatic function and erythropoiesis Ann Int Med 1 677 1927-28
- <sup>1</sup> VON OETTINGEN W F On the relation between the chemical constitution and pharmacological action of phenylhydrazine derivatives J Indust Hyg & Toxicol 18 1 1936
- <sup>1</sup> BRATLEY F G BURROUGHS H H HAMILTON D M AND KERN C The effect of Pyrodon poisoning on the blood and hematopoietic system with special reference to the formation of Heinz Ehrlich bodies in vivo and vitro Am J M Sc 182 597 1931
- <sup>1</sup> GIFFIN H Z AND ALLEN E V Experiments with phenylhydrazine I Studies on the blood Ann Int Med 1 655 1928
- <sup>1</sup> BASSETT, S H KILLIP T AND McCANN W S Mineral exchanges of man III Mineral metabolism during treatment of a case of polycythemia vera J Clin Investigation 10 771 1931
- <sup>1</sup> GIFFIN H Z AND CONNER H M The untoward effects of treatment by phenylhydrazine HCl JAMA 92 1505 1929
- <sup>1</sup> WINTROBE M M Relation of variation in mean corpuscular volume to number of reticulocytes in pernicious anemia The significance of increased bone marrow activity in determining the mean size of red corpuscles J Clin Investigation 13 669 1934
- <sup>1</sup> ROSSCHER ROBBINS F S and Whipple G H Infection and intoxication Their influence upon hemoglobin production in experimental anemia J Exper Med 61 6-7 1936
- <sup>1</sup> CARTWRIGHT G E LAURISTEN M A HUMPHREYS S JONES P J MERRILL, I M AND WINTROBE M M The anemia associated with chronic infection Science 103 72 1946
- <sup>1</sup> WINTROBE M M GREENBERG G R HUMPHREYS S R A HENRICKER H WORTH W AND KRAMER R The anemia of infection III The uptake of radioactive iron in irradiated and in pyridoxine-deficient pigs before and after a uterine inflammation J Clin Investigation 13 1041



# FETAL AND ADULT HEMOGLOBINS IN THE BLOOD OF INFANTS AFFECTED WITH HEMOLYTIC DISEASE OF THE NEWBORN

By ERIC PONDER, M D , D Sc , AND PHILIP LEVINE, M D

A CONSEQUENCE of a preferential hemolysis of red cells containing fetal Hb by the Rh agglutinin-lysin system (Jonxis<sup>6</sup>) should be an increase in the proportion of adult Hb in the blood of infants affected with hemolytic disease, and this proportion should be even greater if, as Jonxis believes, the affected infant develops adult Hb in utero as a protective mechanism. As pointed out by Baar,<sup>1</sup> the conclusions of Jonxis are in contradiction with the results of Baar and Hickmans and of Baar and Lloyd<sup>2</sup>; these results were obtained, however, before the relation of the Rh factor to hemolytic disease of the newborn was appreciated. An independent determination of the proportions of fetal and adult Hb in the bloods of normal newborn infants and of infants affected with hemolytic disease of the newborn may therefore throw light on the situation.

The proportion of fetal and of adult Hb in a mixture of the two can be determined by methods which measure the rate of denaturation of Hb by alkali, the human fetal type denaturing slowly and the adult type rapidly. The method of Brinkman, Wildschut, and Wittermans<sup>3</sup> and that of Brinkman and Jonxis<sup>4</sup> require special apparatus, and Baar and Lloyd's<sup>2</sup> simpler modification of Horowitz's spectrophotometric method requires a photometer more sensitive than that usually available. A procedure which makes the determination of the amount of alkali-resistant Hb much less dependent on the sensitivity of the photometer involves the precipitation of denatured alkaline globin-hematin with half-saturated ammonium sulfate at the isoelectric point.

## METHOD

Red cells are obtained from cord blood, washed, and hemolyzed by freezing and thawing. Sufficient water is added to make a 10 Gm./100 ml. solution of hemoglobin. A volume of 0.2 ml. is added to each of five beakers, each containing 10 ml. of NaOH-glycine buffer of pH 12.15 at 26°C. (4 vols. 7.5% glycine plus 5.85% NaCl per liter and 6 vols. of 0.1 N NaOH). After 10, 20, 40, 60, and 80 minutes, 10 ml. of saturated ammonium sulfate containing enough N HCl to neutralize 10 ml. of the NaOH-glycine buffer is added to each mixture in succession. The alkali-globin-hematin precipitates are removed without delay by filtration with suction (filtering speed at least 10 ml./min.) and the five concentrations of Hb remaining are found either colorimetrically or photometrically as a percentage of the initial Hb concentration. A photometer such as the Lumetron is quite sensitive enough. The logarithms of the concentrations of Hb are plotted against time, except for the point corresponding to the shortest time; they usually fall on a straight line which, when extrapolated to zero time, gives the percentage of fetal Hb present in the mixture.

This method has been tested in two ways:

1. The percentages F of fetal Hb found by the method as applied to normal cord blood have been compared with those found by Baar and Lloyd's photometric method, in which the percentage of denatured Hb present in a mixture at any time is computed from the extinction coefficient of the mixture and the extinction coefficients of Hb and of alkali-globin-hematin. The logarithms of these percentages

From the Nassau Hospital, Mineola, N. Y., and the Rh Blood Testing Laboratory, Ortho Research Foundation, Raritan, N. J.

when plotted against time again fall on a straight line when the times are greater than about 10 minutes and the extrapolation of this line to zero time gives the percentage of fetal Hb originally present in the mixture. Table 1 shows the slope  $d(\log F)/dt$  of this line in 4 cases together with the slope of the line obtained by the method in which the denatured protein is precipitated at pH 7. It also shows the values of  $F_0$ , the percentages of fetal Hb present and found from the intercepts of the lines on the log F axis.

The largest discrepancies between the results of the two methods is usually associated with the values of F for 10 minutes the Baar and Lloyd method tending to give higher values. This point and points for shorter times however are not included in the drawing of the straight line since they lie on a curve which turns upward from it the points corresponding to longer times usually lie very well

TABLE 1

	Precipitation at pH		Baar and Lloyd's method.	
	$d(\log F)/dt$	$F_0$ per cent	$d(\log F)/dt$	$F_0$ per cent
1	0.0036	87	0.0044	88
2	0.0038	85	0.0035	88
3	0.0036	70	0.0046	75
4	0.0048	87	0.0044	94

TABLE 2

	1 vol. with F = 81 A = 19 plus			
	0.2 vol. A = 100	0.5 vol. A = 100	1 vol. A = 100	2 vol. A = 100
Calculated				
Found	67.5 67	54.0 51	40.5 44	27.0 25
	1 vol. with F = 72 A = 28 plus			
	0.2 vol. A = 100	0.5 vol. A = 100	1 vol. A = 100	2 vol. A = 100
Calculated				
Found	60.0 64	48.0 50	36.0 35	24.0 21

on the line irrespective of the method used to obtain them. Since both are extrapolation methods an agreement to within  $\pm 5$  per cent in the final value of F can be considered satisfactory.

2. In several experiments one volume of cord blood containing F per cent of fetal Hb and A per cent of adult Hb was mixed with 0.2, 0.5, 1, and 2 volumes of blood containing adult Hb only (A = 100 per cent). The method was then tested by using it to find the percentage of fetal Hb present in the mixture. Table 2 illustrates the extent of the agreement between the values found and those calculated and shows that the method is applicable over a wide range of concentration of fetal Hb.

## RESULTS

The proportions of fetal and of adult Hb were found by this method in the cord blood of 15 normal full-term infants and of 15 infants affected with hemolytic disease of the newborn. The red cells of the affected infants were coated in the case of the newborn. The reaction with Coombs' serum varying from + to +++++. In the instance of normals the average percentage of fetal Hb was 75 with a standard

deviation of  $\pm 3.9$ , in the group of affected infants, the average percentage of fetal Hb was 78.6, with a standard deviation of  $\pm 4.1$ . There being no significant difference in the percentage of fetal Hb in the two groups, nothing to support Jonxis' conclusions was found.\*

#### SUMMARY

A method is described by means of which the rapidly denatured adult Hb can be separated from the slowly denatured fetal Hb by denaturing with alkali and precipitating the denatured material at its isoelectric point. When applied to 15 normal cord bloods and to the cord bloods of 15 infants affected with hemolytic disease of the newborn, the method showed no significant difference in the percentage of fetal Hb present.

\*Determinations of the quantity of fetal Hb have also been carried out at pH 12.7 and no significant difference has been found between the average values for 7 normal cord bloods and the cord bloods of 7 infants affected with hemolytic disease.

#### REFERENCES

- <sup>1</sup> BAAR, H. S. Fetal hemoglobin and erythroblastosis. *Nature* 162, 190, 1948.
- <sup>2</sup> — AND HICKMAN, E. M. Alkali denaturation of oxyhemoglobin, hemoglobin, carbon-monoxide hemoglobin, methemoglobin, and cyanmethemoglobin. *J. Physiol.* 100, 3P, 1940.
- <sup>3</sup> — AND LLOYD, T. W. Studies on the anemias of infancy and early childhood. Part XIV. The fate of transfused erythrocytes. *Arch. Dis. Child.* 18, 124, 1943.
- <sup>4</sup> BRINKMAN, R. AND JONXIS, J. H. P. The occurrence of several kinds of hemoglobin in human blood. *J. Physiol.* 85, 117, 1935.
- <sup>5</sup> — WILDSCHUT, A. AND WITTMANN, A. On the occurrence of two kinds of hemoglobin in human blood. *J. Physiol.* 80, 377, 1934.
- <sup>6</sup> JONXIS, J. H. P. Fetal hemoglobin and erythroblastosis. *Nature* 161, 850, 1948.

# THE COINCIDENCE OF MEDITERRANEAN ANEMIA AND PERNICIOUS ANEMIA IN A YOUNG SICILIAN

By WILLIAM H. CROSBY (MAJOR, MC, AUS) AND HARRY J. SACKS  
(CAPTAIN, MC, AUS)

**P**ERNICIOUS anemia is ordinarily a disease of the middle years and old age. In individuals under 30 years of age, it is uncommon but not remarkable.<sup>1</sup> Mediterranean anemia particularly in its mild forms (target-oval-cell trait or thalassemia minor) is fairly common, occurring in about 6 to 8 per cent of all Italians studied.<sup>2</sup> The simultaneous occurrence of both pernicious anemia and Mediterranean anemia in one person has not previously been described. We report this association in one case, in which opportunity for careful studies of the blood and bone-marrow was afforded.

## CASE HISTORY

The patient, an enlisted man on duty at a fire station was born at Houston, Texas, July 19, 1920 of Sicilian parents. Family history was negative for blood diseases. The patient's mother died in childbirth while he was a boy. His father, one brother and one sister were known to have diabetes mellitus. One brother was killed in action in World War II. Four other siblings are living and well. The patient had the usual childhood diseases and except for minor illness had been well before the onset of his anemia.

The patient enlisted in the Army Air Forces in September 1946. In December 1946 he developed a severe and intractable diarrhea after eating the fruit of a cactus plant. This persisted with six to twelve watery stools per day for a month. Toward the end of this bout he felt weak, easily fatigued and breathless on moderate exertion. When he fainted on two occasions he went to his dispensary. On January 30, 1947 he was hospitalized at the Station Hospital, Fort Worth Army Air Field.

Physical examination on admission to that hospital showed a patient with a pallid skin, a yellowish tinge and pale mucous membranes. The tongue was very smooth. No other abnormalities were elicited. Blood pressure 110/70.

Laboratory work was reported as follows: RBC 3.0 million, hemoglobin 10.5 Gm, color index 1.0. The blood smear demonstrated macrocytes and tailed erythrocytes. The leukocyte count was 5700. Except for a shift to the right in neutrophils, the differential count was normal. Gastric analysis showed no free hydrochloric acid. The feces were negative for blood and parasites. Urinalysis and serologic test for syphilis were negative. Therapy with liver extract was begun on February 14. Reticulocyte count on February 21 was 3.5 per cent. Polychromasia was noted. On March 3 the blood picture was as follows: RBC 4.0 million, hemoglobin 14 Gm. There were many macrocytes and tailed erythrocytes. Some erythrocytes had basophilic stippling. The blood platelets were 495,000, the WBC 8,800. Liver extract was discontinued on March 5.

The patient was transferred to Brooke General Hospital on March 11. Physical examination on admission was unchanged from that reported earlier. The patient seemed pallid and listless. He was slender with dark complexion, hazel eyes and a sprinkling of gray hairs. There was no evidence of morbilli line to his gums. There was no remarkable lymphadenopathy; the liver and spleen were not palpable. Neurologic examination was negative. The blood counts were essentially unchanged from that of March 3. The patient was group O, Rh negative. There were no sickle cells. Hypertonic fragility was slightly decreased, 0.40-0.31. The blood serum was negative for cold agglutinins. Feces were repeatedly negative for blood, parasites and excessive fat. Gastric analysis with histamine was repeatedly negative for free hydrochloric acid. Urinary urobilinogen was 3.25 mg. per 24 hours. Indirect van den Bergh was 1.0-1.5.

From the Medical and Laboratory Services, Brooke General Hospital, Fort Sam Houston, Texas.

per 100 cc. X-ray of the chest and gastrointestinal system was negative. Proctoscopic examination on April 10 was negative.

A gastroscopy performed on April 22 revealed no evidence of atrophic gastritis\*. A bone marrow examination on April 12 showed no megaloblasts but on May 14 a few megaloblasts were noted with a slight increase in the number of immature erythropoietic elements present.

A short course of liver therapy was given in the attempt to elicit a reticulocyte response. At this time the patient was not anemic. Target cells were first noted in the peripheral blood; however, the patient was returned to duty and examined biweekly as an outpatient. The red cell count at this time was 4.8 million, the hemoglobin 14.7 Gm.

In mid August the patient's red count had fallen and he was readmitted to the hospital. At this time his tongue was sore. There were no neurologic signs or symptoms. The patient complained of easy fatigue and occasional indigestion with belching. Physical examination was unchanged from that of his first admission. The blood picture at this time was: RBC 3.38 million, hemoglobin 13.5 Gm, hematocrit 34 VPC, MCH 40, MCV 101, MCHC 39.7, reticulocytes 0.1 per cent, WBC 4,200, differential normal, platelets 380,000. Anisocytosis and poikilocytosis were evident. Gastric juice contained no free hydrochloric acid after histamine. Urinary urobilinogen 14.1 mg. per 24 hours. Icterus index 8. Indirect van den Bergh 1.4 mg. per 100 cc. Total serum protein 7.0 grams per cent, A/G ratio 2.2. Liver function tests, including cephalin flocculation, thymol turbidity, prothrombin time and bromsulfalein excretion were normal.

A study of aspirated bone marrow at this time revealed erythroblastic hyperplasia. The erythropoietic series consisted chiefly of megaloblastic cells in all stages of maturation; a few cells of the normoblastic series were present. The granulocyte series showed a normal maturation process except for the presence of occasional large hypersegmented neutrophils.

Just before treatment was begun a moderate splenomegaly was noted. The patient was now started on liver extract. There was a well-defined reticulocyte response with a peak level of 20 per cent. The red count rose rapidly from approximately 3.0 to approximately 5.0 million. As the poikilocytosis of pernicious anemia disappeared from the peripheral blood, the target-oval-cell trait became very evident. The patient was discharged from the hospital and from the Army on December 10, 1947.

## DISCUSSION

*Pernicious anemia.* Until this patient was studied the second time we were reluctant to make a diagnosis of pernicious anemia. It was suggested that the macrocytic anemia might have been nutritional following protracted severe diarrhea. This was ruled out by the fact of his relapse while on an adequate diet. The spruelike syndromes were ruled out by a normal small-bowel pattern on x-ray examination and by normal serum calcium and normal fecal fat.

There were many points raised against pernicious anemia. None eliminated the diagnosis but their very number cast suspicion on it. The patient was 26 years old. Pernicious anemia is classed with the degenerative diseases and is seen in the age bracket of arteriosclerosis, cancer and diabetes. Wintrobe<sup>3</sup> reports that of 319 cases of pernicious anemia admitted to Johns Hopkins Hospital from 1925 to 1940, only 4 patients were less than 30 years old.

Our patient was of Sicilian origin. In a study based on admissions to Peter Bent Brigham Hospital, Friedlander<sup>4</sup> reports that 0.16 per cent of all Italians were diagnosed pernicious anemia. The rate for Scandinavians in this same series was 7½ times as great (1.2 per cent), for the English, 5½ times (0.88 per cent).

The gastroscopic examination was negative in our patient. A normal appearing

\* Dr. Don W. Chapman of Baylor University, a member of the civilian professional staff of Brook General Hospital.

gastric mucosa is reported to be found in about 40 per cent of patients with pernicious anemia. Some of those who show atrophy will improve with liver therapy.<sup>6</sup>

It was noted further that in June our patient had been three months without treatment, yet had a normal erythrocyte count. Many cases of pernicious anemia will relapse in this time but some have gone as long as two years without treatment before they relapsed.<sup>6</sup>

There was absence of neurologic changes. vibratory and position sense were intact, there was no paresthesia. Pernicious anemia need not be accompanied by such changes. One of four clinical types of the disease, as classified by Dameshek, is a purely hematologic type, characterized by severe macrocytic anemia and by little if any neurologic involvement.<sup>7</sup>

The target cells found in the peripheral blood could only be explained by supposing there were two diseases. Adding this unlikelihood to the others discussed above we thought it best to make no definite diagnosis.

When the patient returned in relapse, the clinical picture of pernicious anemia was inescapable. There was a macrocytic anemia which responded with reticulocytosis and an increased total red cell count. The bone marrow demonstrated megaloblasts and hypersegmented polymorphonuclear neutrophils. There was absolute achlorhydria after histamine. There was evidence of increased hemoglobin dissolution manifested by a mild acholuric jaundice and increased urinary urobilinogen excretion in the absence of liver disease. There was inflammation and atrophy of the tongue. Lacking only was evidence of central nervous system involvement.

*Mediterranean anemia* When the mask of pernicious anemia with its large and distorted red cells was removed, the target-oval-cell trait became very evident. Mediterranean anemia occurs in degrees of severity varying from the fatal disease which is Cooley's anemia to the target-oval-cell trait, so benign that it cannot be called a disease.<sup>8</sup> The anemia is hereditary, following a mendelian pattern. Its severe form occurs in children whose parents both have a milder form of the disease. The mechanics of transmission have recently been well discussed by Daland and Strauss.<sup>9</sup>

It is of interest that with the simultaneous occurrence of these two diseases, the picture of pernicious anemia dominated. In patients observed with pernicious anemia occurring simultaneously with chronic blood loss the picture was hypochromic.

Our patient returned to Brooke General Hospital for a follow-up examination in February 1948. His blood picture was as follows: RBC 5.88 million, hemoglobin 16.5 Gm, hematocrit 47 VPC, MCH 28, MCV 80, MCHC 35. Cells on stained spreads were slightly hypochromic. The patient was examined at this time by Dr. William Dameshek of Boston who pointed out that hypochromic polycythemia is characteristic of mild Mediterranean anemia. Dr. Dameshek suggested, however, that this diagnosis be confirmed by examination of other members of the patient's family. This was done.

For most of the material we are indebted to the Medical Service of the Veterans Administration in Houston, Texas, which made blood counts and sent blood smears to us for examination.

The patient's wife age 41 Group O Rh positive Irish extraction She was negative for the target oval-cell trait

S B (father of patient) age 63 Diabetic Group A Rh negative RBC 4.35 million hemoglobin 13 Gm Target-oval-cell trait positive

M B N (sister of patient) age 38 RBC 4.53 million hemoglobin 12.5 Gm Target-oval-cell trait negative

J L B (brother of patient) age 33 Diabetic Group A Rh positive RBC 4.39 million hemoglobin 13.5 Gm Target-oval-cell trait positive

J B (brother of patient) age 28 RBC 5.37 million hemoglobin 15 Gm Target-oval-cell trait positive

F B (brother of patient) age 44 Group A Rh positive RBC 4.74 million hemoglobin 14 Gm Target-oval-cell trait negative

M B C (sister of patient) age 20 Severe diabetic Group O Rh positive RBC 4.67 million hemoglobin 14 Gm Target-oval-cell trait negative

P B (daughter of patient) age 3 Group O Rh positive Target-oval-cell trait positive

One maternal uncle and his daughter were negative for target-oval-cell trait

When last examined in March 1948 the patient was in good health. His red cell count was 6.0 million, his blood showed a high proportion of oval cells but only an occasional target cell was observed. When re-examined in November 1948, the patient's physical condition and blood counts were unchanged. He has continued to receive liver injections two to four times monthly.

#### SUMMARY

1. Coincidental Mediterranean anemia and pernicious anemia were found in a 26 year old soldier of Sicilian parentage.

2. The diagnosis of pernicious anemia was made on the finding of achlorhydria after histamine glossitis, megaloblastic bone marrow and macrocytic anemia which responded to liver extract on two occasions.

3. The diagnosis of mild Mediterranean anemia was made by finding the target oval-cell trait in the patient and in five members of his family.

4. It is of interest that target cells were not found in the peripheral blood until treatment with liver was begun. While pernicious anemia dominated, the character of the peripheral blood picture was macrocytic. Liver therapy corrected this, whereupon hypochromic polycythemia characteristic of mild Mediterranean anemia was found.

#### REFERENCES

- <sup>1</sup> SCHWARTZ STEVEN O AND LEGERE HELEN Atypical pernicious anemia of young adults *Am J M Sc* 206 1 1943
- NEEL, JAMES V AND VALENTINE WILLIAM H The frequency of thalassemia *Am J M Sc* 29 568 1945
- <sup>2</sup> WINTROBE MAXWELL M *Clinical Hematology* ed 2 Philadelphia Lea & Febiger 1946 p 354
- <sup>3</sup> FRIEDLANDER R D Racial factor in pernicious anemia *Am J M Sc* 187 634 1934
- <sup>4</sup> HARDT L L SCHWARTZ S O AND STEIGMANN Gastroscopic observations in pernicious anemia. *Gastroenterology* 10 108 1948
- <sup>5</sup> STRAUSS M B AND POHLE F J Duration of remission in pernicious anemia with liver therapy *J A M A* 114 1318 1940
- <sup>6</sup> DAMEHEK WILLIAM *Medical Progress Hematology* New England J Med 232 250 1945
- <sup>7</sup> — Familial Mediterranean target-oval syndromes *Am J M Sc* 203 643 1943
- <sup>8</sup> DALAND G A AND STRAUSS M B The genetic relation and clinical differentiation of Cooley's anemia and Cooley's trait *Blood* 3 438 1948

## EDITORIAL

### INHERITANCE PATTERNS IN MEDITERRANEAN ANEMIA AND SICKLE CELL ANEMIA

IT HAS been amply demonstrated in recent years that the severest form of Mediterranean anemia known as Cooley's anemia, occurs in certain children, both of whose parents show mild forms of the disease<sup>1, 2</sup>

These mild forms are transmitted by either parent in a mendelian dominant fashion and such individuals with the mild target cell or leptocytic disease may be said to be heterozygous for the condition. On the other hand, cases of the severe disease appear to be homozygous for the hereditary trait. The marriage of two heterozygous individuals results (at least theoretically and in accordance with mendelian laws of inheritance) in 50 per cent severe cases, 25 per cent mild or heterozygous and 25 per cent without any evidence of the disease.

Mediterranean anemia and sickle cell anemia show many points of similarity.<sup>3</sup> Both conditions occur primarily in special racial groups, target cells and increased resistance to hypotonic salt solutions are common to both, the anemia does not respond to either the use of iron or splenectomy. Cases of sickle cell anemia have furthermore been described in individuals of Italian and Greek origin<sup>1, 4</sup> and cases of apparently typical Mediterranean anemia are occasionally found in Negroes.<sup>4</sup> As we have previously stated it is possible that both disorders may represent variants of a single larger hereditary abnormality characterized by an abnormal hemoglobin metabolism and defective, unusually thin red cells.

If the heredity of Mediterranean anemia is by now well known, that of sickle cell anemia has, at least until recently, eluded investigation. In line with the inheritance pattern in the former disease, it seemed possible that severe sickle cell anemia might be due to the inheritance of the sickle cell trait from both parents.<sup>2, 5</sup>

The trait itself has been shown to be inherited as a simple mendelian dominant and it seemed likely, therefore, as Neel<sup>6</sup> recently stated, that there existed in Negro populations a gene which in heterozygous condition results in sicklemia and in homozygous condition in sickle cell anemia.

Previous studies of the parents of patients with sickle cell disease had revealed no definite pattern of heredity in fact, in most instances only an occasional parent was found to have the sickle cell trait. On the other hand Neel who tested 42 parents of 29 patients with sickle cell anemia, found that every parent tested to date has sickled.

Neel placed especial reliance for the sickle cell test on a combination of the techniques described by Scriver and Waugh and by Hansen-Pruss. A tourniquet was applied to the finger for three to five minutes and then a drop of static blood from the finger was placed on a slide containing a small amount of Janus green or methylene blue. The preparation was then covered with a coverslip which was sealed with vaseline and examined at intervals up to seventy-two hours. Five preparations were routinely made. It was felt that the variable results obtained



by other observers might be explained in terms of lack of familiarity with the technics necessary to elicit sickling

This important observation will of course require confirmation from other sources before it can receive complete acceptance. However, the results by this experienced geneticist are so clear cut and at the same time so logical that negative results by other workers will, from now on, require considerable scrutiny. That the sicklers are heterozygous and the cases of sickle cell anemia homozygous explains much that has hitherto remained obscure. Nevertheless, the reason for red cell sickling and the exact difference between the red cells of the sickle cell trait and those of sickle cell anemia remain as mysterious as ever.

WILLIAM DAMESHEK

#### REFERENCES

- <sup>1</sup> WINTROBE M M Clinical Hematology Philadelphia Lea and Febiger 1942.
- <sup>2</sup> DAMESHEK WILLIAM Familial Mediterranean target-oval cell syndromes Am J M Sc 205 643 1943
- <sup>3</sup> VALENTINE, W M AND NEEL, J V Hematologic and genetic study of the transmission of thalassemia. Arch Int Med 74 185 1944
- <sup>4</sup> SCHWARTZ S O AND MASON JACK Mediterranean anemia in the Negro A report of four cases and their families Blood 4 706 1949
- <sup>5</sup> NEEL, J V Clinical detection of the genetic carriers of inherited disease Medicine 26 115 1947
- <sup>6</sup> ——— The inheritance of sickle cell anemia Science 110 64 1949
- SCRIVER J B AND WATSON, T R Studies on a case of sickle cell anemia Canad M A J 25 375 1930
- <sup>7</sup> HANSEN PRUSS O G Experimental studies of sickling of red blood cells J Lab & Clin Med 22 311 1936

# ABSTRACTS

JOSEPH F ROSS M D Editor

## ABSTRACTERS

HELEN W BELDINO, M D , Winston Salem, N C  
SHEILA T CALLENDER, M D , Oxford England  
GEORGE E CARTWRIGHT, M D Salt Lake City  
ROGER C CRAFTS, Ph D Boston  
CHARLES P EMERSON, M D , Boston  
SOLOMON ESTREN M D New York  
CLEMENT A FINCH, M D Seattle

OLIVER P JONES, Ph D Buffalo  
CONRAD MAIER, M D , Zurich Switzerland  
MILOS NETOUSEK, M D Prague Czechoslovakia  
JEAN P SOULIER M D Paris  
RAMON M SUAREZ M D San Juan Puerto Rico  
WM N VALENTINE M D , Pacific Palisades, Cal  
JAN WALDENSTROM M D Upsala Sweden

## HEMORRHAGIC DISEASE

TUBERCULOSIS AND STREPTOMYCIN R Dibre H E Brissaud and J P Soulier From the D-partment of Streptomycinotherapy, La Clinique Medicale des Enfants Paris France Sang 29 353-357 1949

Six cases of purpura were observed during one year The patients were treated for tuberculous meningitis or miliary tuberculosis with daily doses of streptomycin varying between 1 and 3 Gm Three purpuras were thrombocytopenic with diffuse hemorrhages Three were athrombocytopenic The relationship between tuberculosis and purpura is discussed In 3 cases the tuberculosis was in an acute phase in 3 others the tuberculosis was no longer progressive

The relationship between streptomycinotherapy and purpura was studied In 3 cases the streptomycin could not be concerned since the treatment had been discontinued eight five and two months before onset of purpura In the 3 other cases cure of the purpura was produced with blood transfusions In 1 case no relapse occurred when streptomycin was restarted In the 2 other cases recovery was complete without having stopped streptomycin Thus there was no apparent relationship between streptomycinotherapy and the mentioned purpuras

J.P.S

THROMBOCYTOPENIC PURPURA IN TUBERCULOSIS OF THE SPLEEN R Lapp (Medical Clinic of the University of Lausanne) Schweiz med Wschr 78 980 81 1948

The author describes 4 cases in which tuberculosis of the spleen was followed by thrombopenia Increased number of megakaryocytes are found in the bone marrow Splenectomy is recommended with protective streptomycin therapy

C.M

RESISTANCE OF THE BLOOD CLOT IN HEMOPHILIA J Král From the 1st Medical Clinic, Charles University Prague Cas lék čes 87 401 1948

Resistance of the blood clot is undoubtedly one of the best diagnostic signs in hemophilia being very low and even equal to zero in this disease In 2 patients suffering from hemophilia the author obtained a definite rise of the zero resistance by injecting 2 cc of rabbit serum This rise continued for several hours after the injection and was accompanied by an increase of red blood cells and blood platelets In both hemophilia and by a reduction of the clotting time The subcutaneous injection of the rabbit serum did not seem to convey any factor lacking in the hemophilic blood it probably developed some unknown mechanism affecting the resistance of the blood clot

M.L.N

PSEUDOHEMOPHILIA OR CHRONIC THROMBASTHENIA C W McLaughlin Jr From the Department of Surgery University of Nebraska Medical College Omaha Nebraska Arch Surg 51 635-645 1947

Two cases classified as pseudohemophilia are reported and the syndrome is compared with hemophilia (von Willebrand 1926) and chronic hereditary hemorrhagic thrombasthenia (Glanzmann 1926)

are discussed. The various means of controlling hemorrhage in pseudohemophilia are mentioned. In pseudohemophilia the bleeding tendency is said to become less marked with advancing years. Surgical treatment is contraindicated unless absolutely required.

WNV

**CAPILLARY FRAGILITY STUDIES (GÖTHLIN TEST) ON ONE HUNDRED PATIENTS RECEIVING DICUMAROL.** R. A. Jubelirer and H. I. Glueck. From the Department of Internal Medicine, The Jewish Hospital, Cincinnati, Ohio. *J. Lab. & Clin. Med.* 34: 448-457, 1949.

One hundred patients receiving dicumarol were studied to determine if any correlation existed between the occurrence of hemorrhage and increased capillary fragility as measured by the Göthlin test. Six of these patients had received dicumarol continuously; the shortest period was three months and the longest, nineteen months. None of these patients demonstrated a positive Göthlin test. Hemorrhage was observed seven times in 100 patients receiving dicumarol. In none of these was the Göthlin index positive. Seven patients demonstrated a positive Göthlin test and gave no clinical evidence of hemorrhage. G.E.C.

**EFFECT OF VITAMIN P LIKE SUBSTANCES ON CAPILLARY RESISTANCE IN THROMBOCYTOPENIC PURPURA IN RATS.** L. O. Randal and E. L. Stirlinghaus. From the Pharmacology Department, Hoffmann-La Roche Inc., Nutley, New Jersey. *Arch. Biochem.* 22: 132-138, 1949.

The decrease in capillary resistance produced by antiplatelet serum as measured by a skin suction test in rats was found to be prevented in part by flavonoid materials and also by certain hydroxy substituted compounds and quinones not obviously related to vitamin P. The test was therefore not specific for vitamin P like materials. Rutin was found much less effective per weight of dose than certain other materials with vitamin P activity. Ascorbic acid and  $\alpha$ -tocopherol phosphate were inactive in preventing the fall in capillary resistance produced by antiplatelet serum.

WNV

**EFFECTIVENESS OF DICUMAROL PROPHYLAXIS AGAINST THROMBOEMBOLIC COMPLICATIONS FOLLOWING MAJOR SURGERY. A FOUR YEAR SURVEY, 3304 CASES.** W. D. Wise, F. F. Loker and C. E. Bamber. From the Department of Surgery and the Department of Clinical Biochemistry, Mercy Hospital and the University of Maryland School of Medicine, Baltimore, Maryland. *Surg., Gynec. & Obst.* 88: 486-494, 1949.

This study of a large series of patients following major abdominal or pelvic surgery adds to the rapidly accumulating data demonstrating a statistically significant reduction in the incidence of postoperative thromboembolic complications with prophylactic anticoagulant therapy in those groups of patients in which the expected incidence is high.

The authors discuss the advantages of chemoprophylaxis over venous ligation, the advantages realized by a conservative rather than drastic reduction of prothrombin activity, and the necessity for rigid standardization of laboratory procedures.

HWB

## LEUKOCYTE MORPHOLOGY AND PHYSIOLOGY

(Studies of Blood Cells with the Phase Microscope Using the Shadow Method for Normal and Leukemic Cells.) **ETUDE SUR LES CELLULES SANGUINES AU MICROSCOPE A CONTRASTE DE PHASE ET PAR LA METHODE DE L'OMBRAOE (AVEC UNE ETUDE PARTICULIERE DES MEGAKARYOCYTES).** M. Bessis. *Rev. Hemat.* 4: 294-349, 1949. **ETUDE SUR L'ETALEMENT DES LEUCOCYTES DU SANG HUMAIN AU MICROSCOPE A CONTRASTE DE PHASE ET PAR LA METHODE DE L'OMBRAOE.** M. Bessis and M. Brila. *Rev. Hemat.* 4: 350-363, 1949. **ETUDE SUR LES CELLULES DES LEUCEMIES ET DES MYELOMES AU MICROSCOPE A CONTRASTE DE PHASE ET PAR LA METHODE DE L'OMBRAOE (AVEC UNE ETUDE PARTICULIERE DES CORPS DE AVEZ ET DE LA FORMATION DE CELLULES DE RIEDER).** M. Bessis. *Rev. Hemat.* 4: 364-395, 1949.

An exhaustive study of blood cells is made with the phase microscope illustrated by 176 microphotographs.

Successively the erythrocytes, the granulocytes, the lymphocytes, the thrombocytes and the megakaryocytes are studied. The structure of the megakaryocytes and thrombocytes seems to be identical.

thus confirming the relationship between these cells. The technic and the interpretation of the preparations are discussed. The normal polynuclears spread out well on plexiglass form but leukemic granulocytes do not spread as well. Polynuclears seen in severe infections spread out especially well on these artificial areas.

The structure of the spread out polynuclear is studied with the electron microscope. It appears identical to that described for the halo-plasm of thrombocytes.

In leukemic cells Auer corpuscles are very easily detected. In one case they were so numerous that it was possible to separate them by mechanical destruction of the cells.

The cells of myeloid and lymphoid leukemias and of myelomas are successively studied.

J P S

**INFRA RED SPECTROSCOPY WITH THE REFLECTING MICROSCOPE IN PHYSICS, CHEMISTRY AND BIOLOGY** R. Barr, A. R. H. Cole and H. W. Thompson. From the Department of Human Anatomy, Oxford, and the Physical Chemical Laboratory, Oxford, England. *Nature* London 163: 198, 1949.

The development of a reflecting microscope by Burch has widened the whole field of microscopy. An important extension of its use to include infra red spectroscopy is now reported. Among the examples given of the application of this technic is the spectrum of a crystal of anti-pernicious anemia factor isolated by Lester Smith. As a whole the spectrum did not show the general features of a polyamide. If it eventually proves to be so, the spectrum must be masked by other parts of the molecular structure. Another line of work which may prove of great hematologic interest is the study of infra-red spectral absorption of biologic cells. There seems every reason to hope that still greater powers of resolution may be obtained with further development of reflecting microscopes so that individual parts of cells may be studied.

S C

**THE HYPERSEGMENTATION OF NEUTROPHIL LEUKOCYTES** J. Kadry. From the Institute of General and Experimental Pathology and the 3rd Medical Department, Masaryk University, Brno, Československo 602 00.

Among 1000 cases of pathologic individuals 48 had hypersegmentation of neutrophils in their blood smears. Besides the commonly known occurrence of these cells in pernicious anemia and other diseases mentioned in the literature, the author calls attention to their very frequent presence in gastric neoplasms. After splenectomy, a considerable hypersegmentation appeared as a temporary phenomenon which disappeared after a certain time.

M. N.

**TRANSPORTATION OF THE ANAPHYLACTOGENIC PROPERTY BY EOSINOPHILS** Z. Z. Godlowski. From the Departments of Pathology and Pharmacology, Edinburgh University, Edinburgh, Scotland. *Brit J Exper Path* 29: 511-524, 1949.

Hypereosinophilia of a local or general character is the most constant feature in allergic conditions and therefore the eosinophil may play an important role in antigen-antibody interaction. Eosinophils were recovered from guinea pigs after an anaphylactic peritonitis had been produced by the injection of horse serum and egg white. The dosage of antigen was estimated from total protein nitrogen values. Various sera, eosinophilic and leukocytic antigens were tested for activity on sensitized guinea pig uterus (Schultz-Dale Test). Evidence has been presented to support the contention that the contraction of the sensitized uterus was precipitated by a specific agent carried by the eosinophil. Other leukocytes do not have this property. The author suggested that eosinophils may transport the antigen to the site of interaction between antigen and antibody.

O P I

**VARIATIONS IN WHITE BLOOD CELLS FOLLOWING THE ORAL ADMINISTRATION OF GLUCOSE TO DIABETIC AND NONDIABETIC** J. W. Jailer, D. T. Marks and P. A. Marks. From the Departments of Medicine, Anatomy and Obstetrics and Gynecology, College of Physicians and Surgeons, Columbia University, and the Presbyterian Hospital, New York, N. Y. *J Clin Endocrinol* 10: 1074-1080, 1950.

The administration of 100 Gm. of glucose in the form of a glucose tolerance test was given to 10

following patients 14 nondiabetics 15 diabetics 1 with Addison's disease 1 with Cushing's syndrome, and 1 with Simmonds' disease. The effects on the absolute lymphocyte count were determined. The normal patients and the three with Addison's disease Cushing's syndrome and Simmonds' disease all reacted similarly with an 18.3 per cent drop in lymphocytes. The diabetic patients showed a 43.2 per cent drop in lymphocytes. The authors found no correlation with the sugar curves. The only exceptions to the above findings were two psychoneurotic cases.

R.C.C.

THE CORRELATION OF THE CIRCULATING POLYMORPHONUCLEAR LEUCOCYTES (NEUTROPHILS) WITH THE ADRENAL ASCORBIC ACID IN THE RAT. *A. Day*. From the Department of Physiology, The George Washington D. C. Endocrinology 43 336-348 1948.

The purpose of this work was to determine whether there was any correlation between the amount of ascorbic acid in the adrenals and the number of circulating neutrophils. Sprague Dawley rats were used. In the normal rats the number of neutrophils decreased as the amount of ascorbic acid increased, thus a negative correlation. The lymphocytes showed no such correlation. Injections of adrenalin decreased the ascorbic acid content of the adrenals but did not influence the neutrophils or the lymphocytes in a similar way. Twenty hours after the adrenalin injection the correlation already mentioned for the normal animal was re-established. Urethane induced a lymphopenia but did not alter the circulating neutrophils or the ascorbic acid content of the adrenals. The authors suggest that the adrenal cortex regulates the number of circulating neutrophils to some extent.

R.C.C.

THE ADMINISTRATION OF ADRENOCORTICOTROPIC HORMONE TO NORMAL HUMAN SUBJECTS. THE EFFECT ON THE LEUCOCYTES IN THE BLOOD AND ON CIRCULATING ANTIBODY LEVELS. *P. H. Herbert and J. A. de Vries*. From the McGill University Clinic, Royal Victoria Hospital and the Department of Bacteriology McGill University Montreal Canada. Endocrinology 44 259-273 1949.

Circulating antibody levels in normal human subjects were not increased following the administration of 40-400 mg. of adrenocorticotrophic hormone. The findings of other workers, that the lymphocyte and eosinophil counts would decrease under such stimulation, were confirmed.

R.C.C.

ON THE FUNCTION OF MONOCYTES IN INFLUENZA VIRUS PNEUMONIA. *Kurt Ballenweiz* (I. Med. Universitätsklinik Charité Berlin). Ztschr. ges. Inn. Med. 1948 437-444.

A cell system is described which develops from inactive prestages of adventitial histiocytes in the medium sized vessels of the lungs under the influence of the irritating action of virus pneumonia. The cells are histiocytic monocytes characterized by the capacity of storing trypan blue in the living state, and by a peculiar arrangement and form within the granulation tissue. Pathologic forms of monocytes and segmented leukocytes in the peripheral blood are described. The vital staining reaction of this cell system is differentiated from the known properties of the reticulo-endothelial system of the liver.

C.M.

## PHYSIOLOGY OF COAGULATION

THE STABILITY OF AC GLOBULIN AND OF PROTHROMBIN IN CITRATED HUMAN PLASMA. *J. L. Fabry, A. G. Ware and W. H. Seegers*. From the Department of Physiology Wayne University College of Medicine, Detroit Michigan. Surg. Gynec. & Obst. 88 370-372, 1949.

Daily determinations were made of the concentrations of prothrombin and Ac globulin in stored citrated plasma and stored citrated whole blood. Prothrombin analysis was carried out by both the two-stage method and a modified two-stage method in which an optimum amount of Ac globulin is provided in the first stage.

The prothrombin content of citrated plasma stored at 5°C. remained constant for at least three weeks as determined by the modified method, whereas plasma Ac globulin concentration remained constant for seven days and then gradually decreased to about a third of the initial level by the third week. This

decrease in plasma Ac globulin approximated the apparent decrease in prothrombin level as determined by the unmodified two-stage method. The stability of prothrombin was unaffected by the presence of cellular elements but Ac globulin was found to be somewhat less stable in whole blood than in centrifuged plasma.

It would appear therefore that blood obtained from hospital blood banks contains its original concentration of prothrombin and that for ordinary use the decrease in Ac globulin is not sufficiently great to be of clinical significance.

H.W.B.

A SIMPLIFIED TECHNIQUE FOR THE DETERMINATION OF PROTHROMBIN TIMES. P. G. Schwager and L. B. Jaques. From the Saskatoon City Hospital and the Department of Physiology University of Saskatchewan, Saskatoon, Saskatchewan. *Canad. M. A. J.* 60: 258-261, 1949.

A simple method for determination of prothrombin time, which has been found satisfactory in the regulation of patients on dicumarol therapy during a two year period is described. In this procedure, whole blood is added at the time it is drawn to thromboplastin. The method was standardized in terms of per cent of prothrombin by taking blood in silicone and preparing a series of red cell-plasma mixtures containing varying dilutions of plasma. (The plasma diluent is not stated.) Preparation of thromboplastin for the individual determinations is described.

This method is recommended for use in office practice and smaller hospitals. In view of the limitations of prothrombin determination as done by generally accepted methods, however, the advantages of simplicity, rapidity and elimination of the necessity for recalcification in this test would appear to warrant its further investigation in dicumarol treated patients.

H.W.B.

THE USE OF RUSSELL VIPER VENOM AND LECITHIN AS THROMBOPLASTIN IN THE ESTIMATION OF PROTHROMBIN. C. A. Mason. From the Pathological Laboratory, Royal Berkshire Hospital, Reading, England. *J. Lab. & Clin. Med.* 34: 458-472, 1949.

Estimations of prothrombin in dicumarin plasma have been compared using the two-stage procedure of Warner, Brinkhous and Smith (modification of Herbert), the one-stage method of Quick, and a one-stage method in which Russell viper venom and lecithin were used as the thromboplastin. The two-stage method gave results in fair agreement with those obtained with the one-stage viper venom method. When rabbit brain or ox lung was used the prothrombin concentration was found to be lower than that given by the other two methods. Using the venom-lecithin method the authors found that hemorrhage was unlikely if the plasma prothrombin was kept above 30 per cent of the normal value.

G.E.C.

ENZYME STUDIES ON HUMAN BLOOD. III. EFFECT OF PLASMA PROTEINS ON COAGULATION. G. Y. Shimamura. From the Department of Pathology, College of Medicine, The Ohio State University, Columbus, Ohio. *J. Lab. & Clin. Med.* 34: 477-481, 1949.

In a previous publication the author demonstrated that the clotting time of a thrombin-fibrinogen system became elevated as the purity of the fibrinogen preparation increased. This observation prompted the author to study the effect of albumin fraction II, III, fraction IV, and hemoglobin on the thrombin-fibrinogen reaction time. It was found that albumin definitely lowers fractions II, III and IV, and fraction IV-4 and hemoglobin slightly depress the clotting time in a system of fibrinogen fractions in citrate-phosphate buffer.

G.E.C.

ENZYME STUDIES ON HUMAN BLOOD. IV. INTERRELATION OF HEPARIN AND FIBROGEN FRACTIONS. G. Y. Shimamura. From the Department of Pathology, Ohio State University College of Medicine, Columbus, Ohio. *Am. J. Physiol.* 154: 458-464, 1949.

The effect of heparin on fibrinogen fractions prepared by low salt low temperature-ethanol procedure was studied. As small an amount of heparin as 0.01 mg. without added cofactor had a measurable coagulant effect on 100 cc. of Seitz filtered fibrinogen fraction. On fibrinogen fraction solutions which were not filtered, variable but reproducible effects were obtained. These varied with the concentration of

of added heparin. The author concludes that this phenomenon suggests the presence of another factor (P) which is necessary for the production of coagulant effects of heparin in fibrinogen fractions.

R.C.C.

**A PROTAMINE TITRATION AS AN INDICATION OF A CLOTTING DEFECT IN CERTAIN HEMORRHAGIC STATES**  
J G Allen P V Moulder R M Elghammer B J Grossman C L McKen M Sanderson W Eger and J M Crosby From the Department of Surgery and the Argonne National Laboratory of the University of Chicago Chicago Illinois J Lab & Clin Med 34 473-476 1949

A method for the determination of heparin like substances in blood is described in detail. This method is based on the fact that when heparin is added to blood and the clotting time begins to increase very small increments of heparin then markedly prolong the clotting time and when these increments are plotted against the clotting times the curve obtained for normal blood is a typical first order curve. Thus in order to make use of the more sensitive portion of the curve blood specimens were made incoagulable by the addition of a standard amount of heparin and then back titrated with a standard solution of protamine sulfate to a clotting end point. Theoretically the amount of protamine sulfate required in such a system to re-establish coagulation would vary in accordance with the concentration of the native heparins and antiheparins of the sample other factors being normal. The protamine requirement under standard conditions was found remarkably constant in both man and dog. Sources of error and the limitations of the method are discussed. In a future publication the authors plan to present their results in certain hemorrhagic states using this method.

G.E.C.

## LEUKEMIA

**CHRONIC LYMPHATIC LEUKEMIA. A STUDY OF 100 PATIENTS TREATED WITH RADIOACTIVE PHOSPHORUS**  
J H Laurence B V A Law-Ber and J W J Carpenter From the Radiation Laboratory and Divisions of Medical Physics (Donner Laboratory and the Department of Physics) and Radiology University of California Berkeley Calif J A M A 140 585-588 1949

The authors are impressed with the ease and satisfactory perhaps encouraging results of the treatment of patients with chronic lymphatic leukemia by means of internal irradiation with  $P^{32}$ . The dosage used was 1 to 2 millicuries per week for from four to eight weeks repeated subsequently whenever the disease relapsed. There was a small increase in the average duration of life under this treatment as compared with the use of x ray alone.

S.E.

**SPONTANEOUS REMISSION IN ACUTE LEUKEMIA. REPORT OF A CASE COMPLICATED BY ECLAMPSIA**  
R F Borg A L Jenks Jr and S K Davis From the Departments of Pathology and Internal Medicine Iowa Methodist Hospital Des Moines Iowa J A M A 140 589-592 1949

The authors collect 11 cases of spontaneous remission of acute leukemia in the literature of which 4 were in children (all females) 3 in adult males and 4 in adult females. The duration of remission ranged from 2 to 24 months (authors' patient). One case showed two separate remissions. In all cases as well as the authors' the eventual outcome was death.

The authors detail the story of the eleventh case a woman of 33 who developed acute leukemia in the seventh month of pregnancy and who at the same time developed eclampsia. One month after the delivery of a stillborn baby the patient had spontaneously improved to a point at which there was complete normality of physical examination blood count and bone marrow smears. She was well for the following twenty-one months when she rather suddenly relapsed and within three weeks died despite treatment. Autopsy showed leukemic involvement of bone marrow and spleen.

S.E.

**FORMATION OF CRYSTALS IN THE CORNEA DURING URETHANE MEDICATION OF MYELOMA**  
N Markoff (Medical Department Hospital of Chur Switzerland) Schweiz. med. Wchnschr. 73 987-988 1948

Deposition of crystals in the cornea occurred during urethane medication of myeloma. As formation of crystals may be found in different organs in myeloma the author asks whether these crystals are so called myeloma crystals or urethane crystals. The fact that they disappeared when the urethane medication ceased seems to speak in favor of the latter.

C.M.

## SPLEEN

A CONSIDERATION OF THE BANTI SYNDROME *P F Wagley* From the Department of Medicine The Johns Hopkins University and Hospital Baltimore Md Bull Johns Hopkins Hosp 83 87-114 1949

Banti's syndrome is reviewed in terms of history laboratory data pathogenesis splenic pathology clinical course and therapy One hundred and thirty three references to the literature are included The author concludes that although splenic vein hypertension has been frequently seen in this condition and may be associated with a variety of abnormalities in the splenic and portal vasculature, the role such hypertension plays other than leading to the formation of gastrointestinal varices and subsequent blood loss is unexplained The evidence for the two most commonly suggested roles of the spleen—namely (1) indiscriminate phagocytosis and (2) humoral inhibition of marrow hemopoiesis is reviewed Therapy is discussed from both the medical and surgical aspects No detailed comparison of results of portal shunt procedure with the procedure of simple splenectomy is made

W N V

PRIMARY SPLENIC PANNHEMATOPENIA *R W Heinle and W D Helden* From the Departments of Medicine and Surgery, University Hospitals of Cleveland and Western Reserve University Cleveland Ohio Surg Gynec & Obst 89 79-91 1949

Seven patients with splenomegaly hyperplastic bone marrow neutropenia anemia and thrombocytopenia were studied before and after splenectomy Evidence that the anemia was clearly hemolytic in nature was lacking With the exception of one patient who died soon after splenectomy from other causes all showed marked although in most instances gradual improvement following splenectomy Examination of the spleens revealed varying degrees of follicular hyperplasia Splenic phagocytic activity of any significance could not be demonstrated in supravital Wright stained or fixed tissue preparations

The authors believe that the findings in primary splenic pannerhematopenia are best explained by the theory that the spleen has some regulatory action on the bone marrow Doubt is cast on the concept that congenital hemolytic anemia and idiopathic thrombocytopenic purpura are members of this syndrome

H W B



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# BOOK REVIEW

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*Symposium de Hematologica y Hemoterapia 1948* By J GUASCH A RAICHS C TRINCAO AND R SURINTACH.  
Barcelona Editorial Minguel Servet 1949 522 pages

This book contains seven sections some of them developed at considerably greater length than is customary in this country on the following subjects penicillin in the treatment of neutropenias (recommended) the treatment of kala azar by splenectomy (recommended in certain cases because the spleen acts by depressing marrow activity) elliptocytosis in man (discussed in great detail classified on none too secure grounds into constitutional elliptocytosis and no less than eleven atypical forms and compared with the occurrence of elliptical cells in the camels, the conclusion is that oval red cells in man and in the camels are similar in a superficial way only) leukemia in pregnancy (2 cases with a précis of cases already described) the blood picture in allergy (eosinophilia the most constant feature) the distribution of the Rh factor in Spain (high percentage of Rh negatives in Belgium and in the north of Spain local variations in Spain not statistically impressive) and the administration of drugs etc., via the bone marrow (recommended with many contraindications most of which are already recognized)

Each section has an English summary All the sections are written from an essentially clinical and morphologic point of view and the extensive bibliographies will appeal to those who like discussions to be thoroughly documented

ERIC FONDER

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Dispensary Assistant Surgeon, Presbyterian Hospital, Philadelphia Penna

WALTER BECKER, M D

Resident Department of Surgery, University of Virginia Hospital Charlottesville Va

BERRY CAMPBELL, Ph D

Department of Anatomy University of Minnesota Minneapolis Minn

RICHARD M CHRISTIAN, M D

Fellow in Medicine The University of Rochester School of Medicine and Dentistry Consultant Atomic Energy Project Rochester N Y

CHARLES G CRADDOCK, JR, M D

Assistant Resident in Medicine University of Virginia Hospital Charlottesville Va

SAMUEL L CRESSON, M D

Resident in Surgery, Pennsylvania Hospital Philadelphia Penna

CHARLES L CROCKETT, JR, M D

Fellow in Internal Medicine University of Virginia Hospital Charlottesville Va

R WENDELL DAVIS, M D

Instructor in Pediatrics The University of Rochester School of Medicine and Dentistry Assistant Pediatrician Strong Memorial Hospital Associate Atomic Energy Project Rochester, N Y

THOMAS J DONOVAN, M D

Research Fellow in Medicine Harvard Medical School Boston Mass

J Q EARLY, M D

Assistant Resident University of Virginia Hospital Charlottesville Va

DONALD M ERVIN, M D

Fellow in Medicine The University of Rochester School of Medicine and Dentistry Associate Atomic Energy Project Rochester N Y

JEAN E FAVRE-GILLY, M D

Laboratoire de Pathologie Interne, Faculté de Médecine de Lyon France at present Fellow of the Relations Culturelles Françaises (Section du Ministère des Affaires Étrangères Paris)

WILLIAM W L GLENN, M D

Assistant Professor of Surgery Yale University School of Medicine New Haven Conn

EDWIN E HAYS, Ph D

Biochemical Research Director The Armour Laboratories, Chicago Ill

BYRD S LEAVELL, M D

Associate Professor of Medicine, University of Virginia Medical School Charlottesville Va

VALY MENKIN, M D

Associate Professor of Experimental Pathology, Temple University Medical School (Agnes Barr Chase Foundation for Cancer Research) Philadelphia Penna

H C MEREDITH, Jr, M D

Resident, Department of Internal Medicine University of Virginia Hospital Charlottesville Va

JOHN B MIALE, M D

Laboratorica of the Marshfield Clinic and St. Joseph's Hospital Marshfield Wisc.

J H MILSTONE, M D

Assistant Professor of Pathology Yale University School of Medicine New Haven Conn

JOHN R MOTE, M D

Medical Director The Armour Laboratories Chicago Ill

ARMAND J QUICK, M D

Professor and Director Department of Biochemistry Marquette University School of Medicine, Milwaukee, Wisc.

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Fellow in Internal Medicine University of Virginia Hospital Charlottesville Va

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Assistant Professor of Medicine, The University of Rochester School of Medicine and Dentistry Assistant Physician Strong Memorial Hospital Consultant Atomic Energy Project Rochester N Y

BERNARD ZIMMERMANN, M D

Assistant Resident Surgeon University of Minnesota Hospitals Minneapolis Minn

# BLOOD

## *The Journal of Hematology*

VOL IV, NO 12

DECEMBER, 1949

### THE PROTHROMBIN CONSUMPTION TEST ITS CLINICAL AND THEORETIC IMPLICATIONS

By ARMAND J. QUICK, M D , AND JEAN E. FAVRE-GILLY, M D

IN 1947, a simple procedure was described for estimating the available thromboplastin of the blood, which was named the prothrombin consumption test<sup>1</sup>. It is based on the principle that by determining the prothrombin before and after coagulation is complete, a measure of the plasma thromboplastin that reacts with prothrombin is obtained. By means of this test it was established that little prothrombin is consumed in the clotting of either hemophilic blood or platelet-depleted plasma.

In the original test, blood was allowed to remain one hour at 37 C. after coagulation before the prothrombin of the serum was determined. While satisfactory results were obtained with hemophilic and thrombocytopenic bloods, occasional inconsistencies were encountered that could not be explained. In searching for the cause of these aberrant results, the important finding was made that when normal blood clots in a test tube, all of the fibrinogen is converted to fibrin before a detectable diminution of prothrombin occurs.<sup>2</sup> This leads to the logical conclusion that the fibrin clot, being uniformly dispersed through the mass of blood, presents an enormous adsorbing surface which quickly and effectively removes the nascent thrombin, thereby preventing sufficient accumulation to initiate the chain reaction that is mediated through the labilizing action of thrombin on the platelets. Almost all of the consumption of prothrombin therefore occurs only after the serum has been separated from the clot either mechanically by centrifugation, or spontaneously through clot retraction.

As a result of the observation that prothrombin consumption is markedly influenced by the separation of serum from the clot, the original test was modified in order to control the adsorption factor of fibrin. Instead of waiting one hour after coagulation before determining the prothrombin of the serum, several test tubes, each containing the same volume of blood, were allowed to coagulate. Every fifteen minutes a tube was centrifuged and the prothrombin of the serum determined at once and after fifteen minute intervals. Since the conversion of prothrom-

From the Department of Biochemistry, Marquette University School of Medicine, Milwaukee, Wis.  
The authors wish to acknowledge the cooperation of the Milwaukee County General Hospital, Milwaukee Children's Hospital and the Junior League Blood Center of Milwaukee in their studies.  
This work was supported by a grant from the Division of Research Grants and Fellowships of the National Institute of Health, United States Public Health Service.

bin is very rapid immediately following the break in the intimate contact of the serum with the fibrin reticulum, thrombin will form and accumulate during centrifugation, therefore the prothrombin time done directly will be abnormally short, since it measures thrombin already present plus the amount formed during the test. By adding sodium citrate to the clotted blood just prior to centrifugation, thrombin formation is stopped, and a true prothrombin value in serum is obtained.

### METHODS

The prothrombin consumption test requires the same reagents as the original one-stage method for determining prothrombin.<sup>2</sup> The thromboplastin is prepared from acetone-dehydrated rabbit brain which consistently yields a prothrombin time of 11 to 12½ seconds for normal human plasma.

*Tricalcium phosphate treated plasma (calcium phosphate plasma)* Tricalcium phosphate quantitatively removes component A from oxalated plasma. It does not remove the labile factor nor fibrinogen; therefore calcium phosphate plasma serves as a ready and convenient source of fibrinogen when determining the prothrombin of serum.

Calcium phosphate plasma is prepared as follows. A measured volume of a 0.005 M suspension of tricalcium phosphate (1 cc. for every cc. of oxalated plasma to be treated) is transferred to a test tube. By centrifuging the gelatinous calcium phosphate is packed and the surplus water poured off. The required volume of fresh oxalated human plasma is added, mixed with the adsorbant and repeatedly stirred with a small glass rod for five minutes at room temperature. The calcium phosphate is removed by centrifugation and the clear adsorbed plasma poured into a clean test tube.

*Prothrombin time of serum* The calcium phosphate plasma (0.1 cc.) is mixed with 0.1 cc. thromboplastin and 0.1 cc. of 0.02 M calcium chloride. Into this mixture 0.1 cc. of the serum is blown by means of a serologic pipet and the time required for a clot to form accurately determined.

*The prothrombin consumption test* Blood obtained by venipuncture is distributed in 2 cc. portions to 8 test tubes (100 x 13 mm.). These are placed in a water bath at 37 C. The time required to form a solid clot is noted and fifteen minutes later 0.1 cc. of 0.4 M sodium citrate is added to one tube. This and a second tube are put in an International Clinical centrifuge and spun at full speed for one minute. After an additional half minute required to stop the centrifuge the prothrombin time is immediately determined in the noncitratd serum and then in the citrated serum. For the latter 0.04 M calcium chloride is used. The prothrombin time of the two sera is determined for three consecutive fifteen minute periods. Thirty minutes after coagulation 0.1 cc. of 0.04 M sodium citrate is added to tube 3, which with tube 4 is centrifuged and the prothrombin time of the serum determined immediately and for two additional fifteen minute periods. At the end of forty five minutes following coagulation tubes 5 and 6 are taken out of the water bath; to one sodium citrate is added and both centrifuged and the prothrombin determined. At sixty minutes tubes 7 and 8 are similarly treated.

For ordinary clinical studies it may not be necessary to follow the prothrombin consumption test in 8 test tubes. Three tubes will suffice. The first is centrifuged fifteen minutes after coagulation, the second after thirty and the third after sixty minutes. The prothrombin time is determined in each tube immediately after centrifugation and every fifteen minutes within the first hour after coagulation.

### RESULTS AND DISCUSSION

*Prothrombin consumption in the clotting of normal blood* In table 1 the prothrombin consumption observed in two typically normal healthy subjects is presented. It will be observed that in subject 1, the consumption of prothrombin is considerably slower and less complete than in subject 2. Thus, in the first individual only 50 per cent of the prothrombin was consumed in thirty minutes, whereas 70 was converted in the second during that period. At the end of one hour, the maximum quantity of prothrombin converted in the serum of subject 1 was approximately 85 per cent, whereas 95 per cent was consumed in the serum of subject 2.

Such marked variations in the activation are interesting, because the concentrations of the factors that constitute the prothrombin complex are remarkably constant in normal healthy individuals. It will be important to study these differences in the availability of thromboplastin as determined by the prothrombin consumption in relation to thrombotic tendencies. While it has been postulated since the time of Virchow that hypercoagulability of the blood is one of the triads that causes thrombosis, no reliable evidence can be found for its support. Hypercoagulability as determined by the coagulation time is not only meaningless, but occasionally definitely erroneous, as will be brought out in the discussion of thrombocytopenia. Since the prothrombin consumption test offers a new and

TABLE 1—*The Prothrombin Consumption During and after the Coagulation of Normal Blood*

	Tube	Time after formation of a solid clot			
		15	30	45	60
		Prothrombin time of serum in seconds			
Subject 1	1	6*	16	17½	17
	2	12½†	13½	12½	12
	3		6½*	18	29
	4		15†	16	15
	5			10*	32
	6			14†	14
	7				10*
	8				15†
Subject 2	1	6*	41	43	45
	2	11½†	12	11½	12
	3		11*	55	52
	4		19½†	20	21
	5			17*	18½
	6			29†	31
	7				17½*
	8				18†

\* Prothrombin determined immediately after centrifugation.

† Sodium citrate added to unretracted clot immediately before centrifugation

promising means to determine the thromboplastin factors quantitatively, its value in postoperative or other conditions in which intravascular clotting commonly occurs will be investigated.

*The prothrombin consumption in hemophilic blood.* Evidence has accumulated since the time of Alexander Schmidt that the defect in hemophilia is a lack of thromboplastin. But Addis<sup>4</sup> believed that the coagulation defect in hemophilia was due to a qualitative change in prothrombin which was responsible for its slow conversion to thrombin. Eagle<sup>14</sup> presented evidence showing that the platelets were functionally normal as well as the prothrombin, but that its activation was delayed for which he could offer no adequate explanation. Brinkhous<sup>15</sup> was the first to study quantitatively the rate of prothrombin conversion in hemophilia and to

concluded that reaction was very slow. This he attributed to the sluggishness with which the formed elements of the blood liberate thromboplastin. More recently, he<sup>6</sup> has postulated that hemophilic blood lacks a factor which is required for the lysis of platelets. In contrast to this explanation, Quick has postulated that the agent responsible for platelet lysis is thrombin itself and that the basic defect in hemophilia is the lack of thromboplastinogen. The platelets have been found entirely normal. Their seeming stability is due to the lack of thrombin formation caused by the deficiency of thromboplastinogen. The new concept introduced by Quick is that platelets do not furnish thromboplastin, but the enzyme which activates plasma thromboplastinogen.

TABLE 2.—*The Prothrombin Consumption Time in Hemophilia*

	Coagulation Time Lee White	Tube	Time after formation of a lid clot			
			15	30	45	60
			Prothrombin consumption time in seconds			
Subject 1	15 min	1	12*	12½	12	12
		2	12†	12	12	12
		3		11½*	13	12
		4		13†	12½	12
		5				12½*
		6				12†
Subject 2	45 min	1	9*	9	9	9
		2	12	11½	12	12
		3		8½*	9	9
		4		12†	12	12
		5				9*
		6				12†

\* Prothrombin determined immediately after centrifugation.

† Sodium citrate added to coagulated blood immediately before centrifugation.

A study of the prothrombin consumption of two hemophiliacs is given in table 2. The data are typical. Similar results have been obtained on 20 other hemophilic patients.<sup>6</sup> Oddly, the prothrombin consumption time immediately after the clotted blood is centrifuged becomes fixed and often does not change in twenty-four hours or longer. Frequently the serum prothrombin time is shorter than that of the oxalated plasma and the usual range is nine to twelve seconds. This shortening of the prothrombin time does not occur until thrombin has accumulated, for on adding sodium citrate prior to centrifuging, to the clotted blood, a normal prothrombin of eleven to twelve seconds is obtained.

The diagnostic value of the prothrombin consumption test in hemophilia is obvious. It is particularly helpful in the diagnosis of the disease in very young children who present difficulties in the collection of blood completely free of tissue juice contamination. Often the diagnosis is delayed for months and even years because of failure to obtain a prolonged coagulation. This actually happened in the case of the second patient. The correct diagnosis was not made until he was

nearly seven years old. In one hospital his condition had been diagnosed as purpura and a splenectomy advised.

*The prothrombin consumption in thrombocytopenic purpura.* Since it was found that the removal of platelets from plasma markedly inhibited the conversion of prothrombin, it could be anticipated that a faulty or delayed prothrombin consumption occurs in thrombocytopenic purpura. This was verified with clinical cases by Soulier<sup>7</sup> and by Quick, Shanberge and Stefanini.<sup>8,9</sup> The latter studied one case in which the prothrombin consumption time improved as the platelets increased and clinical recovery occurred and another case in which splenectomy caused a rapid recovery as indicated by the platelet count and the prothrombin consumption test. In those studies the one hour old serum was employed.

TABLE 3.—*The Prothrombin Consumption Time in Thrombocytopenia*

	Platelet Count	Tube	Time after formation of a solid clot					
			15	30	45	60	75	90
			Prothrombin Consumption Time in Seconds					
Subject 1	15,000	1	8*	9	9½	10½		
		2	11	11	11	11		
		3		9*	10½	13		
		4		11½†	11	11		
		5			8*	13		
		6			11†	11½		
Subject 2	12,000				7*	9½	11	11
		1				7*	11	
		2						8½*
		3						

\* Prothrombin determined immediately after centrifugation

† Sodium citrate added to unretracted clot immediately before centrifugation

In the present investigation several cases were studied by the new technic. The results obtained on 2 of these cases are presented in table 3. It is clear that when the platelet count is low, little prothrombin is converted, but the prothrombin consumption time is not as fixed and constant as in hemophilia. It tends to increase as the serum stands. There is apparently a slow conversion of prothrombin which is to be expected if the hypothesis is correct that the platelets liberate the activating enzyme of thromboplastinogen.

Since a close relationship exists between the platelet count and the prothrombin consumption, the latter test complements the platelet count and can probably be substituted for it when the latter is not available. Since the recognition of thrombocytopenic purpura is relatively simple, the test very likely will contribute little diagnostically. It may, however, become helpful in the condition in which a qualitative change in the platelets exist. Such a condition has been postulated but never satisfactorily demonstrated by concrete tests and experiments.

The most important contribution that the prothrombin consumption test has made to the problem of thrombocytopenic purpura is the establishment that a



demonstrable defect in coagulation occurs despite the normal coagulation time. There is good evidence clinically that neither the low platelet count nor the defective prothrombin consumption is responsible for the petechiae, the ecchymosis or the mucous membrane oozing. These are due to damage or dysfunction of the capillaries caused perhaps by a specific agent. It is logical to suppose, however, that the coagulation defect arising from the thrombocytopenia superimposed on the capillary hyperpermeability accentuates the hemorrhagic state.

*The prothrombin consumption in hypoprothrombinemia.* Since the discovery in 1943<sup>10</sup> that prothrombin activity is not confined to one discrete compound, but to several factors which have been designated as components of the prothrombin complex, the problem has become rather complicated. There is a growing agreement that one of these factors diminishes fairly readily on storage, and is not adsorbed by tricalcium phosphate. This agent has been named the labile factor by Quick. The second factor, component A, is adsorbed by tricalcium phosphate, disappears from the blood in vitamin K deficiency, is probably inactivated by sodium citrate, and is diminished in one type of congenital hypoprothrombinemia.<sup>11, 1</sup> The third factor, component B, is least clearly defined.\* Its existence is postulated to explain the type of hypoprothrombinemia that is both congenital and hereditary and in which no deficiency in the labile factor nor component A occurs.<sup>11, 12</sup> Most characteristic in this type of hypoprothrombinemia is the fixed prothrombin level. In one family the prothrombin time is consistently sixteen seconds in the mother and in a daughter and in one son. Recently a second family has been studied in which the prothrombin time is fixed at fourteen seconds and has appeared in three generations.

In table 4 are recorded the prothrombin consumption tests observed in the blood of a patient treated with dicumarol and in a boy suffering from a congenital deficiency of component A. The prothrombin consumption in the latter is strikingly complete. Fifteen minutes after the clotted blood was centrifuged the prothrombin time of the serum increased to 155 seconds which represents 2 per cent of prothrombin activity. In marked contrast, the blood from the patient on dicumarol therapy which had a prothrombin time of twenty five seconds showed a relatively poor conversion of prothrombin during coagulation. These results cannot be satisfactorily explained until more is known concerning the interaction of the various components of the prothrombin complex. Until such information becomes available the results of a prothrombin consumption test in hypoprothrombinemia will be difficult to interpret and the test will be of limited value clinically.

*The relation of prothrombin consumption to hemostasis.* The most remarkable finding that has accrued from this investigation is that in the test tube only a minute amount of prothrombin is converted to thrombin in the coagulation of all the

\* Since the manuscript was submitted it has been shown (Quick, A. J. and Stefani, M. The state of component A [prothrombin] in human blood. Evidence that it is partly free and partly in an inactive or precursor form. *J. Lab. & Clin. Med.* 34: 1203, 1949) that human plasma contains free and an inactive prothrombin. It is probable that component B is concerned with the conversion of the prothrombin precursor to the active state.

fibrinogen of the blood. Even after the clotted blood has remained in the water bath for fifteen minutes or longer, so little prothrombin is utilized that the amount cannot be estimated. It is only after separation of the serum from the clot takes place that prothrombin begins to decrease rapidly. Obviously fibrin, itself, is the most important physiological anti-thrombin. Potentially, 1 cc. of blood can yield enough thrombin to coagulate all the blood of the body. Heretofore, it was difficult to explain how this powerful latent clotting capacity of the blood was held in check. It is now clear that the strong adsorptive property of fibrin not only guards against the accumulation of thrombin, but also prevents the autocatalytic reaction involving the lability of platelets by thrombin from being set in motion.

TABLE 4.—*The Prothrombin Consumption Time in Dicumarol and Congenital (Deficiency of Component A) Hypoprothrombinemia*

	Plasma Prothrombin Time	Tube	Time after formation of a solid clot		
			15	30	60
			Prothrombin consumption time in sec.		
	<i>sec</i>				
Subject (Dicumarol) 1	21	1	14*	28	35
		2	21†	21	20
		3		23*	33
		4		26†	26
		5			35*
		6			38†
Subject (Congenital) 2	19	1	19*	154	--5
		2	19†	19	19
		3		54*	--8

\* Prothrombin determined immediately after centrifugation

† Sodium citrate added to unretracted clot immediately before centrifugation

In the test tube left undisturbed no significant change in the prothrombin concentration occurs until clot retraction takes place. But this phenomenon as observed in a test tube is purely artificial since the walls are rigid and unlike the more elastic walls of a vein. Clot retraction as seen in a test tube has not been demonstrated *in vivo* and, furthermore, its physiological significance is not known. To be sure, Quick in his monograph<sup>12</sup> presented a pen drawing to show how clot retraction might draw in the torn edges of an injured vessel and thus anchor the fibrin clot. Seegers and Sharp<sup>14</sup> apparently were sufficiently impressed with this concept to reproduce it as a color plate. Unfortunately the interval for which the contracts the clot is weak, and it is difficult to see how this mechanism could function in arteriolar bleeding since such a vessel has not enough fluid to permit a weak force to narrow the lumen. It seems fairly certain that no retraction of separation of serum occurs in intravascular clotting and that the conversion of prothrombin to thrombin takes place *in vivo*.

It would be idle even in the light of these new observations to speculate how hemostasis is achieved, but a few possible suggestions can be offered. Platelet accumulation and agglutination at the site of injury is undoubtedly the early response as Zucker<sup>15</sup> convincingly has shown. As soon as platelets disintegrate thrombin is produced and some fibrin must form. As platelets are lysed, a vasoconstrictor is liberated which contracts all the vessels in the affected area, thus sharply localizing the process. A clot enmeshing the formed elements of the blood, including platelets, will form in the traumatized area. Due to the antithrombic action of fibrin, little thrombin becomes available to labilize platelets, therefore the disintegration of these cells is slow, and the liberation of the vasoconstrictor principle minimal but sustained. The possibility that the fibrin clot is more than a mechanical plug cannot be ignored. It is likely that the fibrin reticulum is the means whereby the coagulation reaction is held in check and that by this means the conditions for sustained hemostasis are maintained.

### SUMMARY

The prothrombin consumption test, which originally was carried out on serum one hour after coagulation, is modified. Blood is distributed to several test tubes, and after fixed time intervals, the tubes are centrifuged. The prothrombin of the serum of each tube is determined immediately, and every fifteen minutes within the limits of one hour from the time the blood is taken.

The prothrombin consumption shows considerable variations in normal individuals. In hemophilia and in thrombocytopenia it is very incomplete. In hypoprothrombinemia the prothrombin may be very complete as in congenital hypoprothrombinemia of the Component A deficiency type, or surprisingly incomplete as in dicumarol hypoprothrombinemia. The possible significance of prothrombin consumption in relation to hemostasis is discussed.

### REFERENCES

- <sup>1</sup> QUICK, A. J. Studies on the enigma of the hemostatic dysfunction of hemophilia. *Am J M Sc.* 214: 271, 1947.
- <sup>2</sup> — AND FAVRE-GILLY, J. Fibrin A factor influencing the consumption of prothrombin in coagulation. *Am J Physiol* 158: 387, 1949.
- <sup>3</sup> — The nature of the bleeding in jaundice. *J A M A* 110: 1658, 1775, 1938.
- <sup>4</sup> ADDIS, T. The pathogenesis of hereditary haemophilia. *J Path & Bact* 25: 427, 1910-11.
- <sup>5</sup> EAOLE, H. Studies in blood coagulation: nature of clotting deficiency in hemophilia. *J Gen Physiol* 18: 813, 1935.
- <sup>6</sup> BRINKHOUS, K. M. A study of the clotting defect in hemophilia: The delayed formation of thrombin. *Am J M Sc* 198: 509, 1939.
- <sup>7</sup> — Clotting defect in hemophilia: Deficiency in a plasma factor required for platelet utilization. *Proc Soc Exper Biol & Med* 66: 117, 1947.
- <sup>8</sup> QUICK, A. J. Pediatric aspects of hemophilia. *Pediatrics* 3: 312, 1949.
- <sup>9</sup> SOULIER, J. P. La consommation de la prothrombine pendant la coagulation du sang veineux et du sang capillaire. *Rev d hemat* 3: 302, 1948.
- <sup>10</sup> QUICK, A. J. SHANBERG, J. N. AND STEFANINI, M. The role of platelets in the coagulation of the blood. *Am J M. Sc.* 217: 198, 1949.
- <sup>11</sup> — AND — The coagulation defect in thrombocytopenic purpura. *J Lab & Clin Med* 34: 761, 1949.

- <sup>10</sup> — On the constitution of prothrombin *Am J Physiol* *140* 212, 1943
- <sup>11</sup> — Congenital hypoprothrombinemia and pseudo-hypoprothrombinemia *Lancet* *2*, 379, 1947
- <sup>12</sup> — The congenital types of hypoprothrombinemia. A consideration of the clinical picture and the hereditary pattern *Chicago M Soc Bull* *52* 614, 1949
- <sup>13</sup> — The Hemorrhagic Diseases and the Physiology of Hemostasis Springfield Ill C C Thomas, 1942. Pp 90
- <sup>14</sup> SEEDERS W. H., AND SHARP E. A. Hemostatic Agents With Particular Reference to Thrombin, Fibrinogen and Absorbable Cellulose Springfield Ill C C Thomas 1948
- <sup>15</sup> ZUCKER M. B. Platelet agglutination and vasoconstriction as factors in spontaneous hemostasis in normal thrombocytopenic heparinized and hypoprothrombic rats *Am J Physiol* *148* 275, 1947

# THE CHAIN REACTION OF THE BLOOD CLOTTING MECHANISM IN RELATION TO THE THEORY OF HEMOSTASIS AND THROMBOSIS

By J H MILSTONE, M D

## THE PHYSIOLOGIC REQUIREMENT

IF OCCASION arose to invent a blood clotting mechanism, it might be arranged for the blood to remain fluid in the vessels, yet promptly congeal when mixed with the juice of freshly cut tissue. This would probably work well for small punctures. However, as this mechanism was tested with larger cuts, an unexpected difficulty might appear. As the blood flowed through the break, a coagulated film would be deposited on the cut surface. This layer would seal over the wounded tissue and hinder the admixture of tissue juice with that portion of blood not yet clotted. Therefore, hemorrhage would continue through a passageway lined by freshly clotted blood.

To overcome this difficulty a chain reaction might be introduced. Then, as one layer of blood was clotting, it would incite the neighboring layer to clot. Thereby the coagulation process could be propagated from one layer to the next, and tissue juice would be needed only to exert its effect on the initial layer. The mechanism could then achieve a hemostatic plug which would grow by accretion, and which, in this respect, would resemble the natural plug depicted by Tocantins.<sup>1</sup>

Thus, by teleologic conjecture, we have arrived at some function a chain reaction might serve. Other functions can be imagined. Ordinary chemical reactions, be they stoichiometric combinations or enzymatic, begin rapidly and thereafter slow down. But, when a chain reaction is involved, one of the products accelerates the reaction, with the result that as more product is formed the reaction goes increasingly faster.\* Hence, chain reactions may start with a lag period, but later are apt to proceed explosively. This offers opportunity for control during the lag period, but ensures rapid action when the lag has been passed or overcome.

## THE BIOCHEMICAL MECHANISM

Whatever its prime function, a chain reaction does occur during the coagulation of blood. An experiment in which the clotting process was transmitted through a series of plasma samples was described by Gratia in 1922.\* Once the process had

From the Laboratory of Pathology, Yale University School of Medicine, New Haven, Conn.

This work was aided by a grant from the James Hudson Brown Memorial Fund of the Yale University School of Medicine.

\*The concept of chain reaction was originally introduced in connection with the photochemical combination of hydrogen and chlorine. It has been broadly applied to various series of consecutive reactions which repeat themselves over and over again. When, in addition, the reaction velocity increases as described by Glasstone (Glasstone, S. Textbook of Physical Chemistry, ed. 2, New York: D. Van Nostrand Co., Inc., 1946, p. 1083), then the chain is said to be nonstationary. It is this type which is implied throughout the present discussion. Here the terms chain reaction and autocatalytic effect are used in order to include other possible mechanisms besides a simple autocatalytic reaction, and autocatalytic reactions are regarded as a special group of nonstationary chain reactions.

been initiated in the first tube, each tube of fluid plasma was caused to clot by seeding it with a few drops of serum from the preceding tube. In 1935, Fischer,<sup>2</sup> apparently unaware of Gratia's previous discussion, reported similar serial passage experiments with minor differences in technic and results. Gratia was impressed by the formal resemblance between the propagation of bacteriophage and the propagation of the clotting process with repeated new formation of thrombin. Fischer wrote of blood coagulation as an endlessly transmissible chain reaction.

Although these demonstrations were spectacular, they were not the first indications of the autocatalytic effect. Beginning in the nineteenth century, two complementary approaches have been made to the analysis of coagulation mechanism: (a) Separation of coagulation factors; (b) separation of individual reactions.

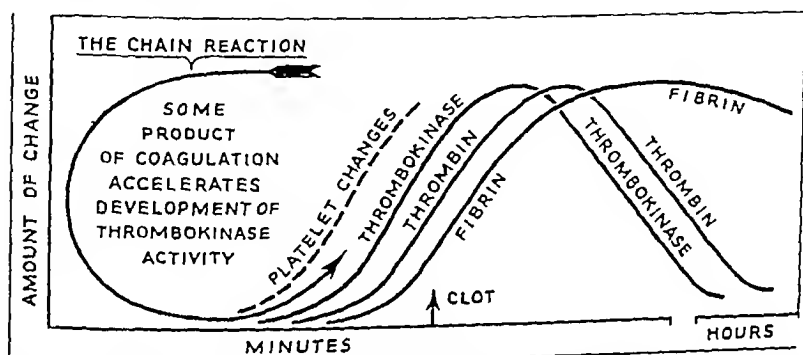


FIG. 1.—SOME EVENTS OCCURRING WHEN BLOOD CLOTS IN A GLASS TUBE. The chain reaction is the kind of process which could cause the growth of a hemostatic plug and the propagation of a thrombus.

The decreases in thrombokinas activity, thrombin and fibrin illustrate the antithrombotic antithrombin and fibrinolytic effects respectively. Such reactions may help to limit the growth of a hemostatic plug or of a thrombus.

This type of synoptic diagram does not appear to have been attempted before and modifications will probably become necessary as more data are obtained. The broken line representing platelet changes is based on observations less pertinent than is the case for the three continuous curves. The historical and experimental background is outlined in the text. (Figure prepared by Mr. Armin H. Rieberger)

By 1904, this twin approach had developed far enough to engender the classic two stage theory

1. Prothrombin  $\rightarrow$  Thrombin In the presence of thrombokinase and calcium
2. Fibrinogen  $\rightarrow$  Fibrin In the presence of thrombin

2. Fibrinogen  $\rightarrow$  Fibrin In the presence of thrombin. But, beginning before 1904, and continuing into the present results have been obtained with separated factors and separated reactions which show that the simple two-stage concept must be modified. And all along the line there have been repeated intimations of autocatalysis. Many studies have been made of the rate at which the coagulation products appear. A frequent finding has been a lag period followed by a period of accelerated production—characteristics of a chain reaction. This is illustrated in figure 1, which outlines the events that occur when blood clots in a glass tube. Actually such a complete experiment has never been

done, and the diagram is a tentative, rough summary of data obtained in various ways

During the performance of the routine test for clotting time, and when the blood is normal, the impression is gained that very little physical change takes place until the end point is imminent. Then, in a comparatively brief interval, the viscosity rapidly increases and a solid clot forms. This gross impression has been confirmed by finer methods, and the production of fibrin is accordingly represented in the diagram. At the time the solid clot appears, less than half the fibrinogen has been converted to polymerized fibrin. As this production of fibrin continues, the clot becomes firmer. After a half-hour or more, the clot retracts. Then, on standing many hours, the amount of the fibrin diminishes somewhat, as can be determined by weighing. This last effect is due to the action of one or more fibrinolytic enzymes, and represents only a partial development of the fibrinolytic potential of the blood. Normally, the enzyme is kept for the most part in an inactive state. As a result of disease or artificial manipulation, fibrinolytic activity can be developed to a surprising degree.<sup>4, 5</sup> It is a remarkable fact that blood normally contains enough potential fibrinolytic activity to liquefy its own clot. For reasons only partially understood, the fibrinolytic potential is rarely, if ever, developed to the full.

The explanation for the shape of the fibrin curve may be complex in detail, but the main reason for its late start is that, up until that time, there is not enough thrombin to clot the fibrinogen. This delay was reported in 1901 by Arthus,<sup>6</sup> who further noted the accelerated production of thrombin just before the clot appeared. Soon the amount of thrombin dwindles—the antithrombin effect.

Carrying the discussion one step further, the delay in thrombin formation is due to the fact that sufficient thrombokinase activity must first be developed. Reasoning from simple experiments on whole blood, Collingwood and MacMahon concluded in 1912<sup>7</sup> that a large part of the clotting time was spent in developing thrombokinase from an inactive precursor which they called prothrombokinase. Recently, by separating the coagulation process into three stages, carried out in three successive sets of test tubes, it has been possible to show that thrombokinase activity develops as shown in figure 1.<sup>8</sup> The latent period and the period of acceleration still suggested a chain reaction, an interpretation corroborated by seeding experiments. There was further noted a prompt and rapid loss of thrombokinase activity, another result foreshadowed by the work of Collingwood and MacMahon.

These are not isolated findings. Using different technical approaches, the interpretations of several recent investigators<sup>9-12</sup> are virtually unanimous on the following broad conclusions: (a) The coagulation mechanism comprises at least three distinct reactions. (b) A chain reaction is involved in the production of a factor that can accelerate the activation of prothrombin.

Contemporary literature, however, shows that these basic findings can be elaborated with greater diversity than might be supposed. The diversity stems partly from the fact that terms like thrombokinase and thromboplastin are used in different ways and that several new terms have been introduced. More

important is the uncertainty concerning which and how many factors participate in the direct activation of prothrombin. Beyond this is the question of which factors, now thought to activate prothrombin, really do something else, such as accelerate the development of thrombokinase. How many different chain reactions occur? To date no convincing evidence has been presented either for or against the occurrence of a simple autocatalytic reaction (e.g.,  $x$  accelerates the production of  $x$ ). Seegers and his associates<sup>15</sup> have brought forth impressive, although not quite conclusive, evidence in favor of a more complicated chain (e.g.,  $x$  accelerates the production of  $y$ , which in turn accelerates the production of  $x$ ).

It is possible to summarize the present situation so as to emphasize the similarities of individual viewpoints rather than their differences.

- 1 Prothrombokinase Complex  $\rightarrow$  Thrombokinase Complex. An autocatalytic or chain reaction results in the acceleration of this conversion.
- 2 Prothrombin  $\rightarrow$  Thrombin. In the presence of the active thrombokinase complex.
- 3 Fibrinogen  $\rightarrow$  Fibrin. In the presence of thrombin.

This is an oversimplification, attained by neglecting details, and by grouping in the thrombokinase complex all factors, including calcium, which may be found to participate directly in the activation of prothrombin.

For the present discussion there are important differences between this formulation and the old two-stage theory. It may be emphasized that the necessary substances for these three stages of blood coagulation are present in, and obtainable from, the blood. The prothrombokinase complex is demonstrably different from the thrombokinase complex, and the conversion from the inactive form to the active form has been followed experimentally.\* This conversion is of further significance in that it consumes a large part of the time required for blood to clot in a glass tube. Moreover, the autocatalytic effect is concerned with the development of thrombokinase activity.\* As a consequence, a small amount of coagulating blood can, when mixed with unclotted blood, accelerate the first of the three reactions, and get the clotting process off to a good start in the fresh portion of blood.

Thus we have rather detailed biochemical evidence of a chain reaction which can take place entirely within the blood, and which can propagate the clotting process.



## THE ROLE OF THE THROMBOCYTES

This advance, although not yet consolidated, is already reaching out to include the thrombocytes. On the experimental side it appears that the chain reaction will proceed in the absence of whole platelets. This does not prove that material derived from platelets is not involved, or that whole platelets are not concerned when they are present. In his review on platelets, Tocantins<sup>16</sup> states the general impression that they will cause changes in the plasma which in turn will lead neighboring platelets to alter, and so on.

The platelet lysis and fusion observed in coagulating blood are brought about not simply by contact with glass in the presence of calcium, but require particularly the presence of a heat-labile factor found in the serum globulins. The serum factor was demonstrated by Wright and Minot in 1917.<sup>17</sup> Brinkhous,<sup>18</sup> on the basis of different considerations, has recognized what may be the same factor, and has suggested the name thrombocytolysin. How this is related to the other factors is not known, but it gives the means whereby the serum can change the platelets. In turn, the platelet material directly or indirectly exerts a pronounced acceleration on the production of thrombin.<sup>19</sup>

A reaction series, shuttling between platelets and plasma, could result in continuously renewed metamorphosis of platelets. Thus a white thrombus might be formed in flowing and eddying blood where fresh plasma and platelets would be supplied continuously to a stationary nidus of metamorphosed platelets. Conceivably this might be independent of the chain concerned in the development of thrombokinase, but there is much to suggest that the two chain effects are intimately related. Exactly how they are related and whether the platelets help or hinder the chain reaction is not known.

A further complexity cannot be ignored. The hemostatic plug, as well as the white thrombus formed in flowing blood, differ appreciably from the test tube clot or the red thrombus formed in stagnant blood. The former have a disproportionately high content of platelets, along with some fibrin (sometimes, apparently very little). Although the fibrin is probably a useful constituent, it is not certain that it is absolutely necessary. Various suggestions that transformed platelets may be sticky even in the absence of fibrin need to be corroborated. In this connection, it is curious that patients with congenital absence of fibrinogen are less incapacitated by their abnormality than is usually the case with haemophilia.<sup>20</sup> Phylogenetic comparisons have suggested that alterations of the thrombocytes represent a primitive component of the hemostatic mechanism, upon which fibrin formation has been superimposed. Be that as it may, even if two adhesive materials are used, it does not necessitate two entirely different mechanisms to apply them. And as yet, the facts do not compel us to postulate a separate chain reaction for platelet metamorphosis.

## THE PROBLEM OF REGULATION

How the chain reaction starts or whether it is always in progress and needs only to be brought to an effective intensity or critical concentration, is still a mystery. We are likewise ignorant of the precise mode of control. It is likely that the control

mechanism exerts not merely a static inhibition, but can also dampen the process when it is already under way. Otherwise, why would not a hemostatic plug or a fresh thrombus continue to grow until it incorporated all the blood in the cardiovascular system?

Several phenomena are known which result in the retardation of the clotting reactions or disposal of their products. Mere dispersion of coagulant products by an active circulation may be of great importance. In the test tube the rapid loss of thrombokinase activity is striking, and here the way is open for its further investigation.<sup>8</sup> If, as Quick<sup>12</sup> and Ware, Murphy and Seegers<sup>14</sup> believe, thrombin is an important link in the chain reaction, then both the antithrombokinase and antithrombin effects offer means for breaking the chain. The fibrinolytic enzyme(s) may accomplish more than is now appreciated. Heparin augments the antithrombin effect, and in some ways delays the activation of prothrombin. Although it is still uncertain whether heparin occurs in significant quantity in normal circulating blood, it appears to reach a highly anticoagulant level after anaphylactic shock and total body irradiation. There have been suggestions that other anticoagulant secretions are discharged steadily or in response to changes in the blood.

Suggestive data on the turnover of platelets, prothrombin and fibrinogen indicate that these factors are completely renewed every few days. It has been inferred that they are consumed in the usual blood clotting reactions, slowly proceeding in the circulation. This implies a degree of dynamic balance to maintain the fluidity of circulating blood, for the converted factors must be removed fast enough to prevent gross coagulation. As illustrated in figure 1 the antithrombokinase and antithrombin effects could take part in this balance. They inactivate coagulant products which have already been formed. Depending on quantitative relations, this kind of action could slow up the clotting process when it was already under way. Such action could help to limit the growth of a hemostatic plug to the requirements of physiologic necessity. The failure, or overpowering of such action might contribute to the excessive propagation of a thrombus.

It is quite possible that the critical juncture of the entire system is the development of thrombokinase activity. But detailed study of this has just begun.

reaction furnishes the biochemical basis for the propagation of a thrombus \* In biochemical experiments the chain reaction has long been in evidence In pathology the formation and propagation of a thrombus has been the subject of classic studies <sup>24</sup> One might speculate what the outcome will be as these data from different disciplines are brought together and extended The chain reaction is so intimately a part of the coagulation mechanism that it is likely to occur whenever a thrombus forms, unless the individual's blood is unusual Consequently, it would usually play some part in the extension of the thrombus The relative importance of its contribution in the genesis of various types of thrombi remains to be evaluated There may be some conditions where a thrombus would propagate even if there were no chain reaction, in some circumstances propagation might be impossible without one Of the latter case, the extension of a mural thrombus far into the chamber of the left ventricle might be an example

Here the growing surface is far from the injured myocardium, and the ventricular blood could hardly be called stagnant Is the continued deposition of platelets and fibrin sustained by diffusion of tissue factor through the thrombus? Or does it depend on repeated formation of fresh coagulant substances at the free surface, through operation of the chain reaction? Anticipation of this question is to be found in the statement of Solandt, Nassim and Best<sup>25</sup> It seems reasonable to suppose that, once agglutinating platelets have covered an injured region, the nature or degree of the underlying tissue damage will have little or nothing to do with subsequent growth of the thrombus The success of their pioneer experiments in suppressing cardiac mural thrombosis by heparinization emphasized anew that the state of the clotting system is very important This may include the chain reaction, but does not yet single it out as the *sine qua non* of cardiac mural thrombosis

#### SUMMARY A WORKING HYPOTHESIS

Detailed evidence has been accumulating that at least one chain reaction occurs during the coagulation of blood Both the metamorphosis of platelets and the development of thrombokinase appear to be involved The autocatalytic effect may serve a function in making possible the growth of a hemostatic plug It also offers advantages in the physiologic control of the clotting mechanism It is likely that the chain reaction occurs in most instances where a thrombus forms, and plays some part in its propagation

The chain reaction is a potentially explosive phenomenon which demands an adequate countermechanism With materials derived from blood, reactions have long since been demonstrated which can reduce thrombokinase activity, inactivate thrombin and liquefy fibrin These reactions may help to maintain the fluidity of the circulating blood by removing the products of smoldering clotting reactions

\* This question has been raised independently in two articles which appeared since the present paper was submitted for publication It was briefly mentioned by B Alexander A deVries and R Goldstein *Blood* 4: 739-746 1949 The idea and a discussion of its implications were presented by J H Mulstone *J Insur Med* 4: 5-7 July 1949 For a step toward the same idea see reference 15 page 74

Such effects could help to delimit the growth of a hemostatic plug, or to end the propagation of a thrombus.

While it is now possible to correlate in this way the data on blood coagulation with present knowledge of hemostasis and thrombosis, critical gaps in our understanding still remain.

## REFERENCES

- <sup>1</sup> TOCANTINS L. M. The mechanism of hemostasis. *Ann Surg* 125 292-310 1947
- <sup>2</sup> GRATIA, A. Discussion on the bacteriophage (bacteriolyisin) IV. *Brit. M. J.* 2, 296 1912.
- <sup>3</sup> FISCHER A. Blutgerinnung als unbegrenzt übertragbare Kettenreaktion. *Biochem. Ztschr.* 279 108-114 1935
- <sup>4</sup> MILSTONE H. A factor in normal human blood which participates in streptococcal fibrinolysis. *J. Immunol.* 42, 109-116 1941
- <sup>5</sup> CHRISTENSEN, L. R. The activation of plasminogen by chloroform. *J. Gen. Physiol.* 30 149-157 1946
- <sup>6</sup> ARTHUS M. Étude sur la production de fibrinogène dans le sang extrait des vaisseaux. *Compt. rend. Soc. de biol.* 33 1024-7 1901
- <sup>7</sup> COLLINGWOOD B. J. AND MACMAHON M. T. The anti-coagulants in blood and serum. *J. Physiol.* 45 119-145 1912.
- <sup>8</sup> MILSTONE, J. H. Three stage analysis of blood coagulation. *J. Gen. Physiol.* 31 301-324 1948
- <sup>9</sup> LAKE K. The autocatalytic formation of thrombin and the clotting defect of hemophilic blood. *Studies Instit. Med. Chem. Univ. Szeged* 3 5-15 1943
- <sup>10</sup> ASTRUP T. Biochemistry of blood coagulation. *Acta Physiol. Scandinav.* 7 Suppl. 21 1944
- <sup>11</sup> OWREN, P. A. The coagulation of blood. Investigations on a new clotting factor. *Acta Med. Scandinav.* Suppl. 194 1947
- <sup>12</sup> QUICK A. J. Studies on the enigma of the hemostatic dysfunction of hemophilia. *Am. J. M. Sc.* 114 272-280 1947
- <sup>13</sup> MILSTONE J. H. Prothrombinase and the three stages of blood coagulation. *Science* 106 546-547 1947
- <sup>14</sup> WARE, A. G. MURPHY R. C. AND SEEGERS W. H. The function of Ac globulin in blood clotting. *Science* 106 618 1947
- <sup>15</sup> SEEGERS W. H. AND WARE, A. G. Protein equilibrium reactions in the blood clotting mechanism. In *Blood Clotting and Allied Problems*. Josiah Macy Jr. Foundation 1 64-84 1948
- <sup>16</sup> TOCANTINS, L. M. The mammalian blood platelet in health and disease. *Medicine* 12 155-260 1938
- <sup>17</sup> WRIGHT J. H. AND MINOT G. R. The viscous metamorphosis of the blood platelets. *J. Exper. Med.* 26 395-409 1917
- <sup>18</sup> BRINKHOF K. M. Clotting defect in hemophilia. Deficiency in a plasma factor required for platelet utilization. *Proc. Soc. Exper. Biol. & Med.* 66 117-120, 1947
- <sup>19</sup> MILSTONE J. H. Activation of prothrombin by platelets plus globulin. *Proc. Soc. Exper. Biol. & Med.* 68 225-228 1948
- <sup>20</sup> WARE A. G. FAHEY J. L. AND SEEGERS W. H. Platelet extracts: fibrin formation and interaction of purified prothrombin and thromboplastin. *Am. J. Physiol.* 154 140-147 1948
- <sup>21</sup> MACFARLANE R. G. Normal and abnormal blood coagulation. A review. *J. Clin. Path.* 1 113-143 1948
- <sup>22</sup> SILVERMAN S. B. A modification of the Waugh-Ruddick test for increased coagulability of the blood and its application to the study of postoperative cases. *Blood* 1 14-154 1948
- <sup>23</sup> HURN M., BARKER N. W. AND MANN F. D. Variations in prothrombin and anti-thrombin in patients with thrombosing tendencies. *Am. J. Clin. Path.* 10 709-711 1947
- <sup>24</sup> ASCHOFF L. Thrombosis. In *Lectures on Pathology*. New York: Paul B. Hoeber, Inc. 1944. Pp. 211-278
- <sup>25</sup> SOLANDER D. J., NASIM R. AND BIST C. H. Production and prevention of a disseminated intravascular coagulation in dogs. *Lancet* 2 592-595 1959

# HYPOPROTHROMBINEMIA STUDIES OF A CASE OF THE IDIOPATHIC TYPE AND THE EFFECT OF SERUM ADMINISTRATION

By CHARLES L. CROCKETT, JR., M.D., DONALD SHOTTON, M.D., CHARLES G. CRADDOCK, JR., M.D., AND BYRD S. LEAVELL, M.D.

**H**EMORRHAGIC diathesis due to idiopathic hypoprothrombinemia was reported first in 1941 by Rhoads and Fitz-Hugh.<sup>1</sup> A case of this type has been admitted to the Pediatric Service of the University of Virginia Hospital five times between 1945 and 1948. In the first portion of this report an account of this case is presented and other similar reports in the literature are reviewed. The second portion of the paper deals with special studies made on our patient which have suggested a new approach to the therapeutic problem presented by patients of this type.

## PART I CASE REPORT

A 5 year old white female was admitted to the Pediatric Service of the University of Virginia Hospital first in November 1945 for study of abnormal bleeding which had occurred intermittently since the age of two weeks. Prior to admission episodes of severe epistaxis, hematemesis and melena had occurred but there was no history of hemarthrosis. On admission hematuria was present.

Developmental history revealed that the patient received an essentially adequate diet but developed somewhat slower than the average child. She experienced no illnesses other than whooping cough and chickenpox and received no salicylates or other toxic medications. A thorough investigation of other members of this family was not possible. No history of hemorrhagic phenomena in other members of previous generations could be elicited from the mother. The prothrombin conversion time of the mother's plasma was normal.

*Physical Examination.* On admission the patient's temperature, pulse and respirations were normal. There were many old hematomata of varying size over both lower extremities but there were no recent hemorrhages. There was no jaundice, adenopathy or hepatosplenomegaly.

*Laboratory Data.* Hematologic studies: red count 3.7 millions, hemoglobin 11 Gm., white count 7,200. Blood and bone marrow differential counts normal. Reticulocytes 1.3 per cent, hematocrit 39, sedimentation rate 6 mm. at the end of one hour. Platelets were 388,000, bleeding time was 2½ minutes (Duke), tourniquet test was negative and clot retraction was normal. The prothrombin time (Quick) ranged from 62 to 92 seconds and the clotting time (Lee White) 11 to 48 minutes on various admissions.

Admission urinalysis showed 3 plus albumin, innumerable red cells, no casts and specific gravity was high. Subsequent examinations were negative. Stool examination revealed *ascaris lumbricoides* ova on the first admission but none on subsequent observations. Blood urea was 20, calcium 9.4. The Schick, tuberculin, Wassermann and plasma salicylate tests were negative.

*Liver Function Studies (1945-1948 inclusive).* Bromsulfalein, hippuric acid excretion, cephalin flocculation, thymol turbidity, proteins and A/G ratio, alkaline phosphatase, cholesterol and esters, icterus index, bilirubin, urobilinogen quantitative 24 hour urine and urobilinogen quantitative fecal were all within normal limits.

*Other Studies (1945-1948 inclusive).* Electrophoretic study of the patient's blood revealed a normal protein pattern with no fibrinogen deficiency. Direct examination of nailbed capillaries revealed normal appearance and normal response to traumatic rupture. Roentgenograms of the chest, skull and long bones were normal.

Deficiency of vitamin K, liver dysfunction and depression of prothrombin by certain substances such as dicoumarol or salicylates were considered as possible causes of the prothrombin deficiency. The lack of

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From the Department of Internal Medicine, University of Virginia Hospital, Charlottesville, Va.

response to large doses of synthetic vitamin K preparations given by oral and parenteral routes seemed to exclude vitamin K deficiency. A careful history examination and numerous tests of liver function failed to indicate liver disease or the presence of any agent known to depress prothrombin formation. Therefore it appeared that this patient had idiopathic hypoprothrombinemia. The absence of any change in either the subjective or objective clinical manifestations including signs of liver disease over a three year period of repeated observations has substantiated this interpretation.

In reviewing the literature on idiopathic hypoprothrombinemia, and the various cases reported therein, one is impressed with the definite disease pattern they seem to form. The onset is during infancy or childhood without any sexual predilection. The family history is frequently bisexually positive for hypoprothrombinemia of a subclinical degree. These patients run a similar chronic course, characterized by cutaneous hemorrhages, epistaxis, hematemesis, hematuria, hemarthrosis, and uterine bleeding severe enough to necessitate hysterectomy.<sup>3</sup> The results of the hemorrhagic tests in these patients are nearly uniform. Besides the prolonged prothrombin times, the clotting time was prolonged in all but the case of Murphy and Clark,<sup>2</sup> which presented more the picture of pseudohemophilia with prolonged and variable bleeding time and abnormal nailbed capillaries, but normal platelets and coagulation time. Though the cases of Rhoads and Fitz-Hugh,<sup>1</sup> and Hagen and Watson<sup>3</sup> showed prolonged bleeding times, these were noted to be only slightly or infrequently abnormal. In general the capillaries, platelets, and other factors have shown no abnormality. The prolonged coagulation time is presumably a reflection of the prothrombin defect, since no increase in circulating anticoagulants, antithrombin or similar substances have been demonstrated.

Successful therapeutic efforts in these cases have been limited to the administration of some effective factor, or factors, present in whole blood, plasma, or as in the authors' case, serum. These patients have been uniformly refractory to various forms of vitamin K administration.

Idiopathic hypoprothrombinemia is not always of a degree sufficient to produce clinical bleeding and several such subclinical cases have been reported. Many of these occurred in families who were studied when one member suffered clinical bleeding, as reported by Giordano,<sup>4</sup> Murphy and Clark,<sup>2</sup> Hauser,<sup>5</sup> and Hagen and Watson.<sup>3</sup> Other cases have been discovered by Plum<sup>6</sup> and Quick,<sup>7,8</sup> and have led the latter to speculate on the prevalence of this state. The prothrombin estimations of these patients ranged from three seconds above the controls to as little as 24 per cent of normal,<sup>4</sup> and in a few the coagulation times were slightly prolonged, but not of the order which those with an hemorrhagic disorder exhibited. The other hemorrhagic tests failed to indicate abnormalities in the other factors concerned in hemostasis, except for a few isolated observations unassociated with any abnormal bleeding.

TABLE 1 —Cases with Chronic Hemorrhagic Disease and Prolonged Prothrombin Time  
(All cases of unknown etiology and all vitamin K refractory)

Authors and year of report	Patient	Age of onset	Family history	Hematologic studies	Prothrombin time and response to therapy
Rhoads and Fitzhugh <sup>1</sup> 1941	18 yr male	9 mos.	Negative	CT 8-360 RT 2-25 CR variable PC normal TT negative and positive qualitative F defect?	Quick 0-123" control 20-24 blood had hemostatic effect
Giordano <sup>4</sup> 1943	22 yr male	5 yrs.	Positive sexual	RT 4 TT positive CT CR PC F normal	Quick 210" Control 25 blood and plasma effective
Murphy and Clark <sup>5</sup> 1944	18 yr male	4 yrs.	Positive sexual	RT 14-15 CT CR PC, TT normal qualitative F defect? nail bed capillaries abnormal	Quick, 60-100" control 16 blood apparently had hemostatic effect
deMarval and Bomchil <sup>11</sup> 1944	14 yr female	8 yrs.	Negative	CT 12-40 RT CR PC F normal TT negative and slightly positive	Quick, 25-55% of normal
deMarval <sup>12</sup> 1945	23 yr female	3 yrs.	Positive	CT 10-12 (abnormal according to author) RT CR PC TT normal	Quick, 20-25% of normal
Hauser <sup>3</sup> 1945	3 yr male	3 mos.	Positive	CT (Bürker) 37"-11 20" usually prolonged RT CR PC, TT F normal	Index, 21-80"
Owren <sup>13a, b</sup> 1947	29 yr female	3½ yrs.	Negative	CT 25 (also prolonged by two other methods) RT 41-5 PC, F normal "Tara hemophilia	Quick, 70-80" control 15-20 blood and factor V (isolated from plasma) effective
Quick <sup>7</sup> 1947	1 yr male	Soon after birth	Positive	CT 12 RT CR PC, F normal Tauc dohypoprothrombinemia	Quick 10", control 11-12.5" blood effective
Quick <sup>7</sup> 1947	5½ yr male	1 wk	Positive Bro of 8	CT 12-13 RT CR PC F normal	Quick 10" control 11-12.5 blood effective
Hagen and Watson <sup>1</sup> 1948	31 yr female	2 yrs.	Positive sexual	CT 60% of tests prolonged RT 43% of tests prolonged mildly CR 40% abnormal TT usually negative PC, F nail bed capillaries normal	Quick, 47-81 controls 11-12.5" plasma effective
Authors	8 yr female	2 wks.	Negative	CT 11-48 RT CR, TT PC, F nail bed capillaries normal	Quick, 55-80" controls 12-15" blood and serum effective

In this table the following symbols are used RT bleeding time CT clotting time CR clot retraction PC, platelet count TT tourniquet test F fibrinogen Quick refers to the one-stage prothrombin method.

period of treatment with vitamin K and blood Autopsy showed a granulomatous process, possibly Hodgkin's sarcoma, and probably hematogenous tuberculosis in the lungs, liver and nodes

In table 1 we have grouped the cases thus far reported in which there is a chronic hemorrhagic disorder associated with a defect in the prothrombin mechanism of undetermined etiology. Thus it can be seen that these cases present a very similar clinical picture and essentially the same laboratory abnormalities if the Quick<sup>1</sup> method of prothrombin determination is used. The various studies indicate that hypoprothrombinemia may not be a single simple defect and that perhaps more than one factor may be involved in deficient prothrombin conversion. Owren<sup>12a, b</sup> concluded that his patient was lacking in what he termed factor V, and chose to designate the resulting hemorrhagic state parahemophilia. Quick<sup>7</sup> studied two brothers with abnormal bleeding and concluded that there was no deficiency of prothrombin component B or the labile factor, but instead, a deficiency of a new coagulation constituent. He felt that pseudo-hypoprothrombinemia was a more fitting designation for this condition. Of course, the implication is that some of these reports are dealing with similar pathologic processes to which different terms or interpretations have been applied.

TABLE 2.—*Prothrombin Determinations on Mixtures of Different Types of Plasma in Equal Volumes (1945)*  
*Plasma Prothrombin Times in Seconds*

Types of plasma added and prothrombin times	Patient	Control	Dicoumarinized	Stored
None	66 0	14 0	87 0	600
Control (14 0)	18 0		14 0	19 0
Dicoumarinized (87 0)*	45 0	14 0		20 0
Stored (Over 600)	25	17	20 0	

\* Prothrombin time of dog's plasma before dicoumarinization eight (8) seconds

## PART II SPECIAL STUDIES

At the time of the first admission of our patient in 1945 certain preliminary observations were carried out in an attempt to determine the nature of the prothrombin defect. Because of the concept of Quick<sup>8</sup> and others<sup>16</sup> at that time that the prothrombin complex was composed of two factors, prothrombin A, which was labile and disappeared from stored plasma, and prothrombin B, which was depressed by dicoumarol, various mixtures of the patient's plasma with normal and old human and dicoumarinized dog plasma (human not available) were set up to determine which component was reduced in this patient. The results are shown in table 2. It will be noted that when stored plasma and dicoumarinized plasma, each with a long prothrombin time, were mixed in equal parts there was a marked reduction in the prothrombin time to a level far below that of either plasma alone. This could be interpreted as indicative of a different type of prothrombin deficiency in each type of plasma and that mixing the two restored the deficient parts of the prothrombin complex more nearly to normal. It was also apparent that the addition of either prothrombin deficient stored plasma or dicoumarinized plasma to the patient's plasma lowered the prothrombin time below that of either plasma alone. This might indicate that the patient was deficient in both of these factors.



An infusion of old plasma brought no appreciable change in the patient's prothrombin time. These data could be interpreted to mean that the patient's plasma was deficient in both prothrombin A and B.

At the time of the admission in 1948 the patient's coagulation defect was felt to be the same as that found on the previous admissions. Possible deficiency of vitamin K was again excluded by the intravenous administration of 75 mg of synthetic vitamin K. The prothrombin time was not affected by this substance, being 63 seconds before injection and 65 seconds 6-12 hours after injection.

The patient's prothrombin time was consistently elevated, ranging from 50 to 80 seconds. These values varied from day to day, despite fair uniformity of activity of the thromboplastin as tested against normal controls. No apparent reason for the wide variations could be found.

In the light of certain recent advances in the knowledge of factors concerned in coagulation, especially in regard to prothrombin conversion, it was felt that some preliminary investigations were indicated to determine the nature of the defect exhibited by this patient. The procedures previously carried out (table 2) involving

TABLE 3 — *Prothrombin Determinations on Mixtures of Different Types of Plasmas in Equal Volumes (1948).  
Prothrombin time in seconds*

Types of plasma added	Patient	Control	Dicoumarinized	Stored
None	55.0	14.2	22.5	35.0
Control (14.2)	17.5		17.3	17.5
Dicoumarinized (22.5)	21.0	17.3		21.5
Stored (35.0)	23.0	17.5	21.5	
Mixture of Dicoum. & Stored (21.5)	19.0	17.0		

mixing of various types of human plasma with the patient's plasma in equal volumes were repeated. The results are shown in table 3.

It was found, as previously, that normal plasma mixed with the patient's plasma in equal amounts substantially lowered the prothrombin time of the patient's plasma, but not to normal levels. Normal plasma mixed with either old plasma or dicoumarinized plasma gave about the same result. Both old plasma and dicoumarinized plasma, each with a prolonged prothrombin time, substantially lowered the patient's prothrombin time, but not as markedly as did normal plasma. Furthermore, it will be noted that a mixture of all three of these abnormal plasmas yielded a lower prothrombin time than any two.

These data could be interpreted as previously to indicate that the lower prothrombin times resulting from mixtures of the various prothrombin deficient plasmas was due to an elevation of the prothrombin concentration of the plasma mixture as a result of different portions of the prothrombin complex being supplied by the various individual plasmas. However, this interpretation, which is according to the concepts of Quick, is open to some objections. Against it is the fact that the addition of purified prothrombin\* to the patient's plasma failed to bring

\* Supplied through the courtesy of Dr. Walter H. Seegers, Wayne University College of Medicine, Detroit, Mich.

the prothrombin time to normal. Bringing the concentration of added prothrombin to 100 mg per 100 cc lowered the prothrombin time from 67 to 37 seconds. The additions of increasing amounts failed further to reduce the time of prothrombin conversion. This result would indicate a deficiency of some other factor necessary for the rapid conversion of prothrombin to thrombin as well as a deficiency of prothrombin per se. Mixing the various types of prothrombin-deficient plasmas may have altered the concentration of this factor and hence caused more rapid conversion of prothrombin, even though the latter was still in low concentration.

The existence of a factor in normal blood which is necessary for the rapid conversion of prothrombin seems to be beyond dispute. The disagreement as to the nature of the factor and its mechanism of action will not be discussed here. We have chosen to use the term accelerator or activator globulin (Ac-globulin) after Ware, Guest, and Seegers,<sup>17</sup> but are cognizant of the unsettled similarity of the substance so named to the labile factor of Quick,<sup>9</sup> factor V and factor VI of Owren,<sup>12a, b</sup> and the prothrombokinase of Milstone.<sup>18</sup>

Because of the importance of this Ac-globulin in the rate of prothrombin conversion it is apparent that the Quick one-stage prothrombin technic is not an accurate measure of prothrombin concentration in the plasma, since the variable factor of accelerator globulin activity is not controlled. The two stage determination<sup>19</sup> of prothrombin concentration would appear to be less affected by variations in accelerator globulin since the rate of conversion of prothrombin is not measured, but rather the actual amount of thrombin produced during a definite incubation period. However, this test may be criticized as a measure of prothrombin concentration since the activity of Ac-globulin apparently also influences the amount of thrombin produced.<sup>20</sup> Seegers, et al. have recently modified the two stage method to measure the concentration of prothrombin by adding known amounts of Ac-globulin to a reaction mixture containing specified amounts of thromboplastin, calcium ions and the plasma to be tested, and incubating for varying periods of time before adding fibrinogen. Although the Quick one stage method<sup>1</sup> does not measure actual prothrombin concentration, it does serve as an accurate measure of the speed of prothrombin conversion to thrombin, a reaction in which all of these factors take part. It will be noted that in the protocols presented, prothrombin is expressed in terms of prothrombin time rather than per cent concentration of normal. This terminology was used because it is evident that a measure of prothrombin time cannot be accurately interpolated to prothrombin concentration by the usual graphic method unless the activity of Ac-globulin is controlled.

Ware and Seegers have demonstrated<sup>20</sup> that accelerator globulin is in a highly active state as it exists in serum after clotting. They believe that it is in a less active state in the plasma where it exists as a proenzyme. According to these authors it is activated by the formation of small amounts of thrombin in the first stage of clotting, amounts too small to cause clotting of fibrinogen. The activation of plasma Ac-globulin is then followed by increased thrombin formation and the reactions proceed by co-autocatalysis. After the concentration of thrombin has been built up to the point where clotting occurs, the reaction is spent. The thrombin formed is destroyed, as is thromboplastin, but Ac-globulin was found to survive in the serum in a highly active state.

It was therefore felt that some estimation of the activity of accelerator globulin might be ascertained by studying the influence of small amounts of the various sera on the speed of prothrombin conversion of the various plasmas. It was considered that the small amounts of other factors pertaining to clotting in thrombin-free serum, such as antihemophilic globulin, platelet breakdown products, and prothrombin would not interfere with the use of serum as a rich source of this material. Platelet breakdown products and antihemophilic globulin appear to participate in the initiation of coagulation and furnish active thromboplastic effect. They have no influence on the rate of prothrombin conversion when an excess of artificial tissue thromboplastin is used, as shown by Ferguson and Lewis.<sup>21</sup> The prothrombin content of fresh serum immediately after clotting is normally less than 10 per cent of that of normal plasma. Barium carbonate adsorption of the residual prothrombin was carried out in some instances but difficulty was encountered in avoiding an excess of the material which inhibits the activity of prothrombin in the plasma to which the barium carbonate-treated serum was added. It was not felt that the small amount of prothrombin in the serum would be sufficient to bring about the striking changes noted.

TABLE 4—*The Effects of Various Sera on the Prothrombin Times of Various Types of Plasma (One Part Serum in Three Parts Plasma) Plasma Prothrombin Times in Seconds*

Types of sera added	Patient	Dicoumarinized	Stored
None	60.5	24.8	23.5
Normal serum	15.7	14.5	17.0
Patient's serum	48.5	19.5	16.5
Dicoumarinized serum	28.5	24.0	23.3
Stored serum	21.0	18.5	21.0

It will be seen from table 4 that one part of normal serum in three parts of the patient's plasma brought about a marked reduction in the prothrombin time from a value of 60.5 to 15.7 seconds (normal control 15.0 seconds). Normal serum also brought about a striking reduction in the prothrombin time of dicoumarinized and old plasma, particularly the former. On the other hand, the patient's serum did not bring about nearly so marked a reduction in the prothrombin time of either the patient's or dicoumarinized plasma, but did reduce that of old plasma, the reason for which is not apparent. Neither serum from dicoumarinized nor stored blood possessed as much accelerating effect as did normal serum. There seemed little doubt that normal serum possessed a factor which was capable of accelerating prothrombin conversion in the patient's plasma. It is apparent that any alteration in prothrombin concentration by the addition of serum could not account for this remarkable reduction in prothrombin time (table 3). This acceleratory effect of normal serum on prothrombin conversion is also demonstrated by the use of purified prothrombin. A clotting mixture consisting of purified prothrombin 0.1 cc., human fibrinogen 0.2 cc., and thromboplastin 0.05 cc. was used. Upon recalcification with 0.1 cc. calcium chloride (0.024 M) at 37°C it was observed that purified prothrombin was converted to yield sufficient thrombin to clot human fibrinogen in an average

time of 286 seconds. This is in accord with previous findings that thrombin is produced slowly, in the absence of Ac-globulin by the interaction of thromboplastin, calcium and purified prothrombin.<sup>2</sup> However, if 0.05 cc. normal serum (thrombin-free) was added to the mixture at the time of recalcification with 0.05 cc. calcium chloride, clotting occurred in 45 seconds. Also it was noted that fresh normal serum possessed a more marked acceleratory effect than did normal serum which was 24 to 48 hours old, the latter bringing about clotting in 74 seconds. In contrast, using the same technic, fresh serum from the patient produced clotting in 90 seconds and 24 hour old serum in 110 seconds.

The data in table 5 show the relative potency of fresh normal serum in accelerating the conversion of prothrombin in the patient's plasma. These findings would be in accord with the concept of the enzymic or catalytic action of this substance.

A modification of the two stage method of prothrombin determination was performed in order to evaluate further the concentration of prothrombin in this patient's blood. The results of this test seemed to indicate an actual deficiency of prothrombin, as well as delay in its convertibility to thrombin, as previously demonstrated. A 1:11 dilution of control defibrinated plasma yielded a prothrombin time of 13 seconds whereas the same dilution of the patient's defibrinated plasma yielded a prothrombin time of 68.5 seconds. However, since adequate control of

TABLE 5—*The Effect on the Prothrombin Time of the Patient's Plasma of Serum Added to Increasing Amounts of Plasma*

Proportions of serum to plasma	1:1	1:3	1:6	1:12	1:20	1:40	1:80	0:1
Prothrombin time in seconds	16	16.5	18	22	25.5	31.5	39	57

accelerator globulin activity was not possible, interpretation of the results was difficult. Dr. Walter Seegers kindly performed determinations of both prothrombin and Ac-globulin of a sample of the patient's plasma. He reported a prothrombin concentration of 41 per cent by the original two stage technic and 60 per cent by the modified technic in which the amount of accelerator globulin was controlled. He concluded that the Ac-globulin concentration was in the neighborhood of 50 to 60 per cent of normal. It is interesting to note that neither the prothrombin concentration nor that of Ac-globulin, as determined by Dr. Seegers, was as low as might have been anticipated. Perhaps the moderate depression of both these factors simultaneously was sufficient to bring about the marked retardation of prothrombin conversion noted, whereas a deficiency of either factor alone would have to be more extreme before a comparable delay in prothrombin conversion time would be noted. Although Dr. Seegers' prothrombin value was somewhat higher than our tests would seem to indicate, his general conclusions, that a deficiency of both factors was present, are in agreement with the results we obtained.

The marked acceleration of prothrombin conversion in the patient's plasma by the addition of normal serum in vitro prompted the administration of fresh normal serum to the patient in the hope of lowering the prothrombin time of the circulating blood. Accordingly a normal donor of the same blood type was bled. The

blood was allowed to clot and the serum removed under sterile precautions. Fifteen cc of this serum was injected intravenously 2 hours after collection, at which time it was free of thrombic activity. There was a slow decline in the patient's prothrombin time from a level of 67 seconds to 37 seconds in 16½ hours. At this time 45 cc more of the serum (which was by this time 18 hours old) was given and the prothrombin time fell from 34 seconds to 25 seconds in one hour. Three hours after the second injection the patient's prothrombin time was 22 seconds. This was the lowest prothrombin time yet recorded for this patient. Twenty-four hours later the patient's prothrombin time was 32 seconds. It is of interest that as little as 15 cc of serum given intravenously had a significant effect. These results are shown graphically in figure 1.

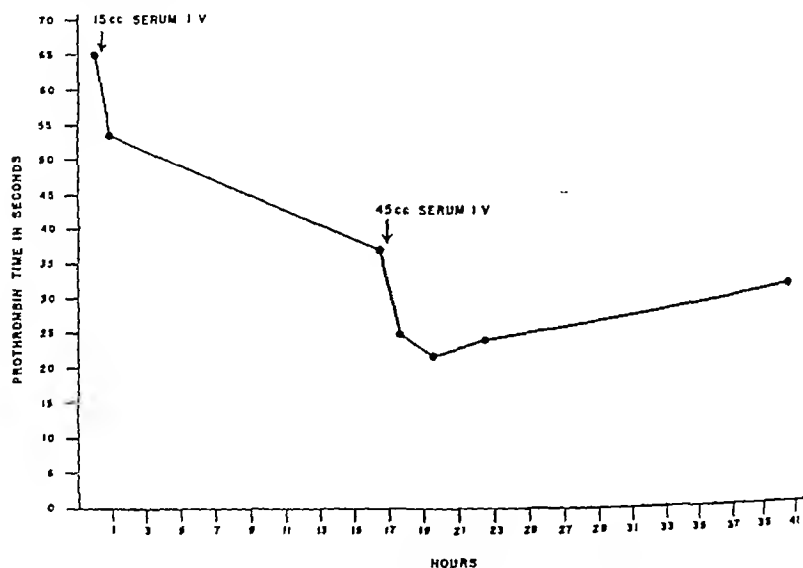


FIG 1 —The effects of intravenous administration of fresh serum on the prothrombin time of patient H H

It is interesting to note that the patient's coagulation time, which was 19 minutes on admission, was now 12 minutes, suggesting an improved state of coagulability of the patient's blood. It is also of some interest that the patient's serum became more active in accelerating prothrombin conversion when added to her own plasma after the above treatment. Thus, one part of the patient's serum obtained after treatment, with three parts of the patient's plasma collected before the intravenous administration of serum, lowered the prothrombin time from 64 seconds to 27 seconds, whereas previously the patient's serum had reduced the prothrombin time from 60.5 seconds to 48.5 seconds.

The patient was seen March 15, 1949 on a return visit after the preceding data had been completed. She was admitted to the hospital because of bleeding from her gums for three weeks. Examination revealed six carious deciduous teeth. The

gingival tissue around some of these was hypertrophied and irritated and bleeding occurred with mastication. Prothrombin time (Quick) was 72 seconds with a control of 12, and the clotting time (Lee White) was 23 minutes. There were no other significant changes from the previous studies.

In figure 2 are shown the effects of giving the patient fresh whole blood and fresh serum, both separately and, later, together. After each administration it will be noted that the prothrombin and clotting times were markedly reduced within an hour's time and further, that the most marked reduction was obtained when the two were given close together, near the end of her stay. These results are interpreted as further evidence in support of the hypothesis that she is deficient

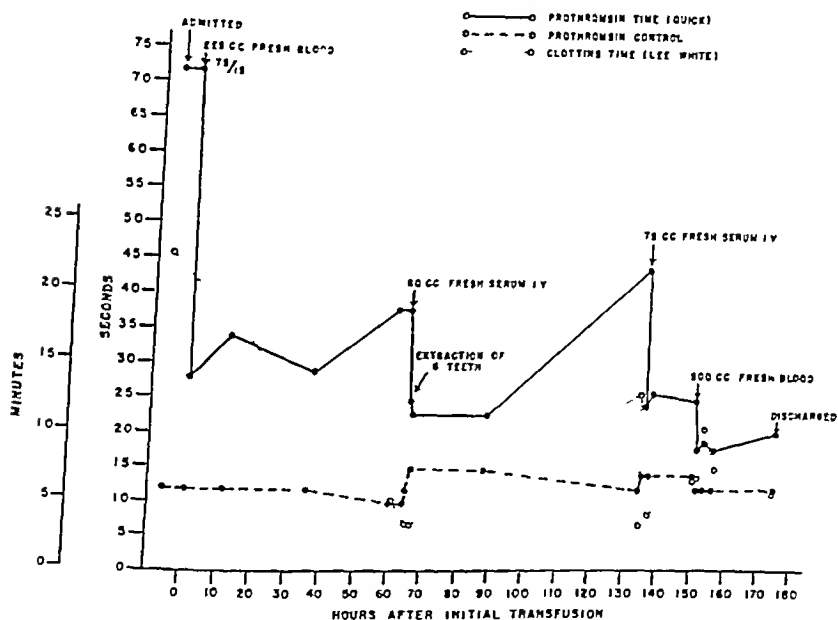


Fig. 2. The effects of fresh blood and serum on the prothrombin and clotting times of patient H. H.

in two factors, prothrombin and accelerator globulin, which were presumably supplied by the blood and serum respectively.

The extraction of the six carious teeth was done under a general anesthetic and bleeding was minimal both in amount and duration. Her course remained uneventful and it was felt that she had been carried through a potentially dangerous procedure by the use of the blood and serum.

### Discussion

These studies support the concept that serum contains an active substance which is capable of accelerating prothrombin conversion to thrombin. It is likely that deficiency of this substance may play a role in many types of hemorrhagic states as suggested by Alexander and co-workers.<sup>23</sup> It is probable that alterations in

Ac-globulin are of particular importance in various types of prothrombin deficiency. Further investigation is needed to delineate clearly the different types of hypoprothrombinemia and to evaluate the use of serum in treating certain cases of this type.

Our observations also suggest, as do those of Owen and Bollman<sup>14</sup> in dogs, that depression of Ac-globulin activity is a major factor in the hypoprothrombinemia produced by dicoumarol. It is obvious that if such be the case, serum or some fraction of the serum such as Ac-globulin may be of potential value in quickly reducing the prothrombin time to safe levels in cases of dicoumarol intoxication. We have carried out preliminary observations on the effect of intravenous normal serum from compatible blood on the prothrombin time of three patients receiving dicoumarol. The pattern of response in all has been the same. In the most recent patient the prothrombin time before giving 175 cc. of serum was 49 seconds. An hour after administration of the serum it was 30 seconds, rising to 38 seconds at the sixth hour, with controls of 15. Twenty-four hours later it had risen to 40 against a control of 12 seconds.

From the evidence that both prothrombin and Ac-globulin are depressed in hypoprothrombinemia following dicoumarol and with liver disease<sup>10a, 15</sup> control of the coagulation defect will depend upon the correction of both deficiencies. An apparently effective and simple means of so doing is to give whole blood, thus supplying prothrombin and fresh, thrombin-free serum rich in Ac-globulin in a highly active form.

#### SUMMARY AND CONCLUSIONS

1. The reported cases of idiopathic hypoprothrombinemia are reviewed briefly, and a case observed for over three years is presented. Particular attention is called to the similar clinical pattern presented by the chronic cases.

2. Studies are presented indicating that in this patient the delay in prothrombin time was due, at least in part, to a deficiency of a factor necessary for rapid conversion of prothrombin. This factor, or factors, which we have called Ac-globulin, is contained in a highly active state in fresh normal serum.

3. After the *in vitro* demonstration of a deficiency of Ac-globulin in the patient's blood, it was possible to bring about a marked reduction in the patient's prothrombin time by the intravenous administration of relatively small amounts (15 to 45 cc.) of fresh normal (thrombin-free) serum. A further reduction of the prothrombin time to near normal values was brought about by combined whole blood and serum administration. The evidence suggests that partial correction of both prothrombin and Ac-globulin deficiency respectively resulted from such therapy.

4. The possible effects of serum and whole blood upon the delayed prothrombin conversion rate of dicoumarolization and liver disease are discussed and preliminary observations in the former type suggest that such therapy may be useful.

#### ACKNOWLEDGMENTS

We wish to express our appreciation to the following for their cooperation in this study: Dr. McLemore Birdsong, Associate Professor of Pediatrics, University of Virginia Medical School.

The Department of Pediatrics University of Virginia Hospital The Department of Biochemistry University of Virginia Medical School Dr Myers H Hickle Assistant Resident in Medicine University of Virginia Hospital Dr Walter H Seegers Professor of Physiology Wayne University College of Medicine

# REFERENCES

- <sup>1</sup> PHOADE J F AND FITZ HUGH T JR Idiopathic hypoprothrombinemia—an apparently unrecorded condition *Am J M Sc* 21 662-670 1941
- <sup>2</sup> MURPHY F D AND CLARK J E Idiopathic hypoprothrombinemia *Am J M Sc* 207 77-83 1944
- <sup>3</sup> HAGEN P S AND WATSON C J Idiopathic (familial) hypoprothrombinemia *J Lab & Clin Med* 33 342-354 1949
- <sup>4</sup> GIORDANO A S Idiopathic hypoprothrombinemia *Am J Clin Path* 13 285-287 1943
- <sup>5</sup> HAUSER F Familiale Vitamin K resistente hypoprothrombinämie *Ann paediat* 165 142-348 1945 from Hagen and Watson <sup>3</sup>
- <sup>6</sup> PLUM P Idiopathic hypoprothrombinemia refractory to vitamin K *Acta Med Scandinav* 113 262-265 1943
- <sup>7</sup> QUICK A J Congenital hypoprothrombinemia and pseudo-hypoprothrombinemia *Lancet* 253 379-386 1947
- <sup>8</sup> — On the constitution of prothrombin *Am J Physiol* 140 212-220 1943
- <sup>9</sup> — Components of the prothrombin complex *Am J Physiol* 151 63-70 1947
- <sup>10</sup> LEWIS J H AND BENNETT I J JR Clinical hypoprothrombinemia A study of factors A and B of prothrombin *J Clin Investigation* 26 1187-1188 1947
- <sup>10a</sup> — Personal communication to Hagen and Watson <sup>3</sup>
- <sup>11</sup> AUSTIN, V T AND QUASTLER H Idiopathic (?) hypoprothrombinemia A Report of a case *Am J M Sc* 210 491-500 1945
- <sup>12</sup> QUICK A J AND LEU M Quantitative determination of prothrombin *J Biol Chem* 119 no 2, LXXXI 1937
- <sup>13</sup> OWREN P A Parahaemophilia Hemorrhagic diathesis due to absence of a previously unknown clotting factor *Lancet* 252 446-448 1947
- <sup>14</sup> — The coagulation of blood *Acta Medica Scandinavica Supplementum CXCTV (194) (accompanies Vol CXXVIII) 1-327 1947*
- <sup>15</sup> DE MARVAL L AND BOMCHIL G Hypoprothrombinemia idiopathica *Semana med* 1 1088-1092, 1944 from Hagen and Watson <sup>3</sup>
- <sup>16</sup> — Hypoprothrombinemia idiopathica *Semana med* 1 476-477 1945 from Hagen and Watson <sup>3</sup>
- <sup>17</sup> ONEAL W J AND LAM C P Experiments on prothrombin *Am J M Sc* 210 181-184 1945
- <sup>18</sup> WARE A G GUEST M AND SEEOERS W H Plasma accelerator factor and purified prothrombin activation *Science* 41-42, 1947
- <sup>19</sup> MILSTONE J H Prothrombokinase and the three stages of blood coagulation *Science* 106 546-547, 1947
- <sup>20</sup> WARNER E D BRINKHOUS K M AND SMITH H P A Quantitative study on blood clotting Prothrombin fluctuations under experimental conditions *Am J Physiol* 114 667-675 1936
- <sup>21</sup> WARE A G AND SEEOERS W H Serum Ac globulin Formation from plasma Ac globulin role in blood coagulation Partial purification Properties and quantitative determination *Am J Physiol* 152 567-576 1948
- <sup>22</sup> FERGUSON J H AND LEWIS J H Accelerator Globulin and Antihemophilic Globulin in thrombin formation from aged prothrombin and in hemophilic blood *Proc Soc Exper Biol & Med* 67 228 1948
- <sup>23</sup> SEEOERS W H BRINKHOUS K M SMITH H P AND WARNER E D The purification of thrombin *J Biol Chem* 126 95-95 1938
- <sup>24</sup> ALEXANDER B DE VRIES A AND GOLDSTEIN R A prothrombin activator in serum and its significance in certain hemorrhagic diseases *J Clin Investigation* 27 523 1948
- <sup>25</sup> OWEN C A AND BOLLMAN J L Prothrombin conversion factor of dicoumarol plasma *Proc Soc Exper Biol & Med* 67 231 1948
- <sup>26</sup> SYKES E M JR SEEOERS W H AND WARE A G Effect of acute liver damage on Ac globulin activity of plasma *Proc Soc Exper Biol & Med* 67 506 1948

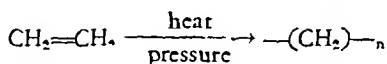


# THE EFFECT OF ARTIFICIAL SURFACES ON BLOOD COAGULABILITY, WITH SPECIAL REFERENCE TO POLYETHYLENE

By THOMAS J. DONOVAN, M D , AND BERNARD ZIMMERMANN, M D

## INTRODUCTION

AS A PRELIMINARY evaluation of the efficacy of polyethylene tubes in reparative vascular surgery, comparative studies were carried out on the coagulability of blood in tubes of polyethylene and other nonvascular materials. Polyethylene is a plastic made by polymerizing ethylene under heat and pressure to hydrocarbon chains somewhat longer than those of paraffin. This may be represented as follows:



This plastic should be well tolerated in body tissues, since synthetic plastics of the simplest monomeric structures are known to cause the least reaction. This was established by Ingraham et al., who found that small pieces of polyethylene implanted in the cerebral cortex of dogs, cats, rabbits and monkeys caused only slight glial thickening with no significant foreign body reaction, up to three months postoperatively.<sup>10</sup> They stressed, however, the importance of using *pure* polyethylene, because any traces of the antioxidants used commercially to increase its insulating properties have given progressive fibrosis and marked foreign body reaction.

The effect of a pure polyethylene surface on blood clotting was investigated in two ways. First, a series of clotting times was performed in tubes of polyethylene, glass, paraffin and collodion. Secondly, the capillary action in polyethylene and glass tubes was studied in an attempt to explain the increased coagulation time in polyethylene tubes.

## MATERIALS AND METHODS

*Effect of polyethylene on tissue.* Inasmuch as the purity of the polyethylene\* is an important factor in the coagulation studies, small pieces of polyethylene and lucite were inserted subcutaneously in the backs of 30 rats and the surrounding tissue removed *en bloc* for histologic study at various intervals. Polyethylene was found to cause the same degree of fibrosis as lucite, a plastic which has been shown to be tolerated relatively well in animal and human tissues. The thin fibrous sheath surrounding the plastic was seen to increase slightly during the first three months. Figure 1 shows the reaction to polyethylene and lucite at three months. The fibrous

From the Naval Medical Research Institute, Bethesda 14, Maryland.

The opinions or assertions contained herein are the private ones of the authors and are not to be construed as official or reflecting the views of the Navy Department or the Naval Service at large.

\* The polyethylene used in these studies was supplied by D. C. Balfour Associates, Englewood, New Jersey, in November 1947.

capsule surrounding the space from which the plastic was removed at fixation showed no foreign body giant cells or leukocytes under higher magnification.

*Measurement of coagula in time.* Glass tubes of 5 mm i.d. were bent in the form of a semicircle of 6 cm radius as nearly identical geometrically with a polyethylene tube as it was possible to make them. Some of the glass tubes were lined with a thin layer of paraffin by filling them with hot liquid paraffin for a moment, emptying the tubes and placing them in a cold water bath for a few seconds. Other glass tubes were lined with a collodion film which was allowed to dry for eighteen hours. Four tubes, one each of glass, paraffin, collodion and polyethylene were

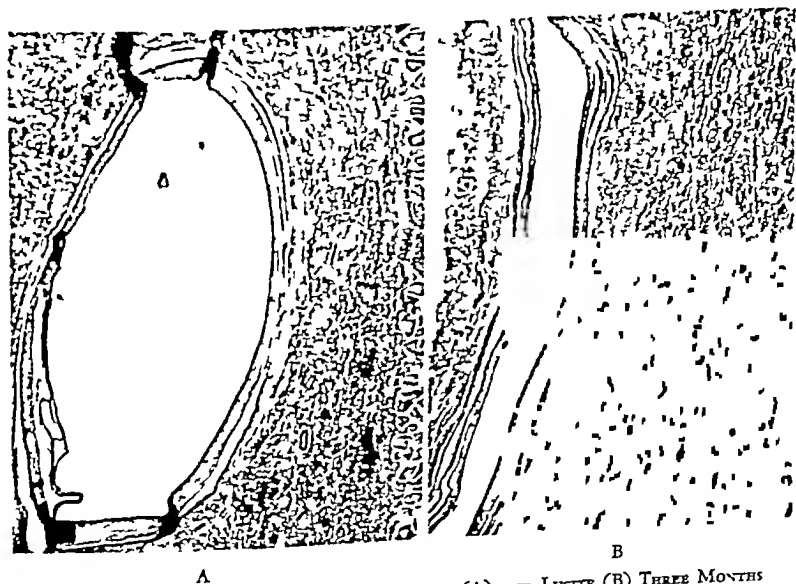


FIG. 1—THE TISSUE REACTION TO POLYETHYLENE (A) AND LUCITE (B) THREE MONTHS AFTER SUBCUTANEOUS INSERTION INTO RATS

The fibrous capsule surrounds the space from which the plastics were removed during fixation. The width of each piece of plastic represented by the diameter of the capsule was about 2 mm while the length approximated 1 cm.

inserted in a rack. Venepunctures were performed on healthy dogs using an 18 B-D needle attached to a 10 cc syringe lined with liquid petrolatum. Ten cc of blood were allowed to flow into the syringe with exclusion of air, the needle was removed, and a little over 2 cc of the sample was transferred carefully into each of the four tubes. The time was noted by stop watch when the first definite fibrin precipitate became perceptible in each tube on moving the rack. The temperature was kept relatively constant at  $20^{\circ}\text{C} \pm 5$  degrees in an air-conditioned room. In the first groups of clotting times, the rack was moved every thirty seconds until the

\* When any difficulty with the venepuncture was encountered the blood was discarded and the procedure repeated.

last tube clotted. In this way each tube was moved uniformly and each group of four observations was comparable to the other fourteen in extent of motion of the blood in the tube. In the last fifteen groups of clotting times the rack was moved at three minutes and at decreasing intervals thereafter as the end points were approached. Thus, the motion of the blood was reduced and the clotting times for each surface were somewhat lengthened. The first fibrin precipitation was used as an end point because it marks the beginning of the second phase of coagulation, i.e., fibrinogen conversion to fibrin. It has been re-emphasized by Nygaard that either this end point or complete clotting is suitable since the first phase of coagulation, i.e., prothrombin conversion to thrombin, is proportional in duration to the second phase.<sup>16</sup> In tubes of various materials complete clotting may be less clearly defined, as an end point, than the initial appearance of fibrin.

*Measurement of surface action.* The amount of adhesive force exerted by the surfaces of glass and polyethylene on water was measured by suspending various sized capillary tubes\* of glass and polyethylene in clean graduates half filled with distilled water in an air-conditioned room maintained at  $25^{\circ}\text{C} \pm 2$  degrees. The differences between the water level in the capillary tube and the graduate were measured by a cathetometer. These measurements were repeated at lengthening intervals until the difference in water levels became constant. In glass tubes such constancy was obtained in minutes, while with polyethylene tubes in the initially dry state the final level was reached in one to two weeks. Since the delay in obtaining a constant level is undesirable, some of the polyethylene tubes were soaked in distilled water for periods up to two and one-half months prior to the determination of their capillary action. This served to increase markedly the initial and final wettability of the polyethylene tubes, and hence the height of the fluid levels, but did not hasten the attainment of a final constant level.

The capillary action of polyethylene and glass tubes was also measured using canine plasma in place of distilled water. Harkins and Brown and other workers have found that organic, viscous liquids of alkaline reaction give exceptionally low surface tension values and are unsuitable for capillary action studies.<sup>7</sup> The values obtained for polyethylene and glass tubes in plasma were markedly low, as would be predicted, but were roughly proportional to those determined for polyethylene and glass using distilled water.

The radius of each tube was determined by filling a carefully measured length of the tube with pure mercury, which was then weighed. From the radius and the distance water is repelled or attracted, a negative or positive value, respectively, for the adhesive force between the surface and water was obtained by the equation

$$T (\text{attractive force in Gm/cm}) = \frac{\text{height} \times \text{density} \times \text{radius}}{2} \quad (1)$$

## RESULTS

*Effect of surface on blood coagulation.* The mean values of the 30 clotting times for each surface studied are shown in table 1, in which they are compared with a

\* Tubes were chosen having a cylindrical bore of about 0.05 cm radius which did not vary significantly in any part of the tube.

similar series reported by Hirschboeck in a comparison of lucite, glass and paraffin.<sup>8</sup> Hirschboeck's clotting times were all longer than those of the present series, because he used as his end point complete clotting instead of the first fibrin deposition. He also used human blood in tubes twice the diameter of ours. Canine blood has been shown to have a greater prothrombin conversion rate than human blood.<sup>11</sup> The differences between the clotting times in glass and paraffin, glass and the plastic tested, and between paraffin and the plastic tested are statistically significant in both series.

These data show that the clotting time in a polyethylene tube is about twice as long as in a glass tube and almost as long as in tubes lined with paraffin or collodion. Comparison of this series with that of Hirschboeck shows that the effect of polyethylene and lucite surfaces in delaying coagulation are very nearly the same and are not greatly inferior to those of paraffin and collodion, which simulate vascular endothelium in this respect as well as any known surface.

Clot retraction was observed repeatedly following the coagulation studies, and although there was some variation, the collodion surface was exceptional for the

TABLE 1.—The Coagulation Times (Minutes) of Blood in Contact with Various Surfaces

Series	Poly ethylene	Lu cite	Glass	Paraf fin	Collo- dion	No. of com parative obser vations	End point	Size of tubes (diam.)	Blood used
Authors	11.5	—	5.3	12.4	12.5	30	Earliest sign of clotting	5 mm.	Canine
Hirschboeck		13.9	6.2	18.3		10	Complete clotting	10 mm.	Human

absence or slight degree of clot retraction. In the paraffin glass and polyethylene tubes retraction was present to a moderate and essentially similar degree.

**Effect of surface on capillary action.** In glass tubes of .056 cm. and .053 cm. radius the water levels were stabilized immediately at heights of 2.62 cm. and 2.73 cm., respectively. These figures substituted in the equation give positive values (uncorrected for meniscus) of 0.073 and 0.072 Gm./cm., respectively, for the attractive force of glass on water at 25°C. These agree well with the value of 0.0736 Gm./cm. at 25°C. calculated from the surface tension value for water at 20°C. established by Harkins and Brown<sup>7</sup> and other workers using the capillary height method. Hirschboeck reported a value of .053 Gm./cm. for the force of adhesion of water in glass in a comparison of the capillary effect in tubes of glass, lucite, paraffin, and collodion.<sup>8,9</sup> No details were presented concerning his methods, but the figure is exceptionally low as compared with the well-established standard value mentioned above.<sup>7</sup>

Figure 2 shows typical capillary action curves for measurements in two different sizes of polyethylene tubes starting from an initially dry state. The polyethylene originally repels water markedly, nearly to the extent reported by Hirschboeck for paraffin.<sup>8</sup> Over a period of days, however, the levels rise at a diminishing rate

and flatten out in about a week. Calculations based on the final levels observed for the two sizes of polyethylene tubes give a value of 0.34 Gm/cm for the attractive force of polyethylene on water at 25 C, slightly under half the above-mentioned value established for glass.

Table 2 shows the values for surface force in polyethylene and glass tubes as compared with the Hirschboeck values for glass, lucite, paraffin, and collodion<sup>6</sup> and the standard value for glass.<sup>7</sup> The maximum attraction of polyethylene for

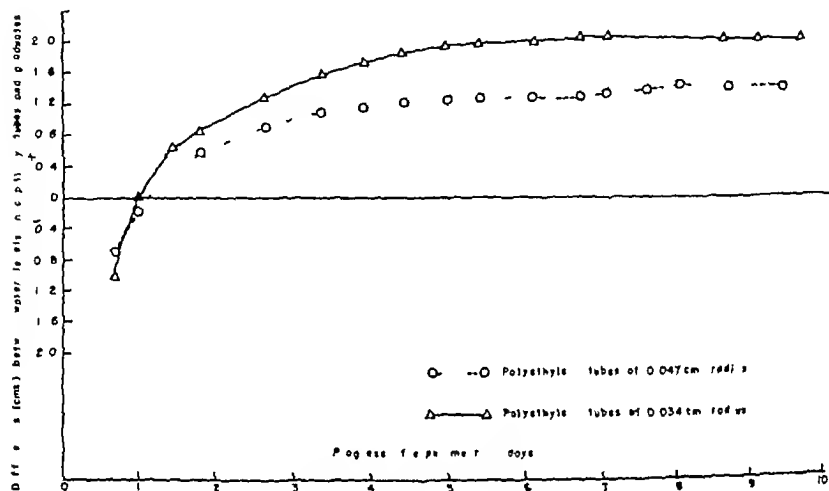


FIG. 2.—CAPILLARY ACTION IN TWO SIZES OF POLYETHYLENE TUBES OVER A TEN DAY PERIOD

TABLE 2.—The Values (Gm/cm) for the Adhesive Force between Water and the Surfaces of Polyethylene and Glass (These figures are compared with Hirschboeck's values for glass, lucite, paraffin and collodion<sup>6</sup> and the established value for glass<sup>7</sup>)

Series	Polyethylene	Lucite	Glass	Paraffin	Collodion
Authors	+0.034	—	+0.073	—	—
Hirschboeck	—	+0.038	+0.033	-0.037	+0.034
Harkins and Brown	—	—	+0.0736	—	—

water as determined above is about half that of glass and slightly less than that reported for lucite. Initially it repels water nearly as much as paraffin.\*

Lampert has stated that the effect of a surface on the coagulation time of blood is inversely proportional to its wettability.<sup>7</sup> Comparison of the data in table 2

\* The application of the capillary height method to surface tension measurements may be considered to be of somewhat doubtful value in systems of liquids which do not wet glass; hence as in these experiments of water against unwettable polyethylene. The angle of contact may safely be assumed to be zero for water against glass, but the same is not true for the plastic systems under consideration. Although no correction has been made for this factor and the values are accordingly not offered as being absolutely correct, they serve the purpose of this work adequately.

with those in table 1 shows that polyethylene follows Lampert's rule, as do lucite, paraffin, and glass with collodion as an exception

### Discussion

The process that determines the speed and extent of coagulation under normal circumstances is the conversion rate of prothrombin to thrombin, which is in turn dependent on the amount of active thromboplastin present. Thromboplastin is released outside of the blood stream from tissue juices and within the vascular system by the breakdown of platelets. Indirect and debatable evidence suggests that thromboplastin is normally present in the circulating blood, but is rendered inactive by combination with an antithromboplastic substance, and that the balance between the concentrations of these substances determines the balance between coagulability and fluidity of the circulating blood.<sup>17, 5, 4, 3, 12, 8, 11, 1</sup>

✓ Surfaces that repel water, such as paraffin and some of the plastics, tend to inhibit the agglutination and disintegration of platelets and the subsequent liberation of thromboplastin.<sup>18</sup> Since the extent of clot retraction is normal in polyethylene tubes, this material, in contrast with collodion surfaces, presumably has no increased affinity for fibrin. Furthermore, polyethylene repels water relatively well and therefore probably inhibits platelet agglutination and lysis in the same manner described by Quick for paraffin and other water repelling surfaces.<sup>16</sup>

The mechanism by which water repelling surfaces delay coagulation, however, may not be concerned solely with platelet disintegration. Lozner and Taylor and others describe a catalytic action of foreign surfaces on the clotting of cell-free recalcified plasma.<sup>11</sup> Tocantins postulates that the increased coagulability of cell-free plasma after exposure to glass is due either to an activation of thromboplastin or an inactivation of antithromboplastin.<sup>17</sup> He cites the work of Gortner and Briggs who reported a negative charge on a glass surface which disappeared when the glass was lined with paraffin.<sup>4</sup> Electrostatic absorption of positively charged colloids was suggested by the latter as a possible mechanism in the initiation of the clotting of blood in contact with glass.

✓ The coagulation time is nearly as long in polyethylene tubes as in tubes lined with paraffin. Polyethylene's chemical structure, clot retraction, and repelling action on water are also similar to those of paraffin. It is therefore suggestive that it delays coagulation in a similar manner, i.e., by its relative inertness to the clotting colloids of blood as well as through its protective action on the stability of platelets.

### SUMMARY AND CONCLUSIONS

1. The reaction to small pieces of polyethylene and lucite in the subcutaneous tissues of 30 rats was studied at intervals up to three months after insertion. Polyethylene was shown to compare favorably in respect to minimum tissue reaction, with the well-tolerated lucite.

2. A series of clotting times was performed in polyethylene, paraffin, collodion and glass tubes. The clotting time in polyethylene tubes was about twice as long as in glass, and nearly as long as in paraffin and collodion-lined tubes. These data are similar to Hirschboeck's findings for lucite tubes.

3 Clot retraction was found to be moderate and essentially similar in polyethylene, paraffin, and glass tubes. It was slight or absent in collodion tubes.

4 Capillary tubes of polyethylene were shown to repel water initially and then gradually to attract water over a period of days to a maximum height about one half of that in glass tubes. Thus polyethylene follows Lampert's rule, which states that the effect of a surface in delaying the coagulation of blood is proportional to the capacity of that surface for repelling water.

#### ACKNOWLEDGMENTS

The authors wish to express appreciation to Drs. H. G. Saroff, J. Sendroy, Jr., J. L. Tullis, E. P. Cronkite, and Miss M. E. Tarver for helpful advice and cooperation in these studies.

#### REFERENCES

- <sup>1</sup> ALLEN, J. G., SANDERSON, M. AND JACOBSON, L. O. Hyperheparinemia—the cause of the hemorrhagic disease produced by total body exposure to ionizing radiation. Atomic Energy Commission Contract with Metallurgical Lab., Univ. of Chicago, Nov. 1946.
- <sup>2</sup> BEST, C. H. Heparin and thrombosis. Harvey Lectures, 36: 66–89, 1940–1941.
- <sup>3</sup> CHAROFF, E. The coagulation of blood. *Advances in Enzymology*, F. F. Nord and C. H. Werkman, New York: Interscience Publishers, Inc., 1945, pp. 31–59.
- <sup>4</sup> — ZIFF, M. AND COHEN, S. S. Studies on the chemistry of blood coagulation. 10. The reaction between heparin and the thromboplastic factor. *J. Biol. Chem.* 136: 257, 1940.
- <sup>5</sup> FERROUSON, J. H. Mechanism of blood coagulation. *Am. J. Med.* 3: 67–77, 1947.
- <sup>6</sup> GORTNER, R. A. AND BRIGGS, D. R. Glass surfaces versus paraffin surfaces in blood clotting phenomena: a hypothesis. *Proc. Soc. Exper. Biol. & Med.* 25: 820, 1928.
- <sup>7</sup> HARKINS, W. D. AND BROWN, F. E. The determination of surface tension and the weight of falling drops. The surface tension of water and benzene by the capillary height method. *J. Am. Chem. Soc.* 41: 499, 1919.
- <sup>8</sup> HIRSCHBOECK, J. S. Delayed blood coagulation in methyl methacrylate (boilable Incite) vessels. *Proc. Soc. Exper. Biol. & Med.* 47: 311, 1941.
- <sup>9</sup> — Delayed blood coagulation and absence of clot retraction in collodion lined vessels. *Proc. Soc. Exper. Biol. & Med.* 45: 122, 1940.
- <sup>10</sup> INGRAHAM, F. C., ALEXANDER, E. AND MATSON, D. D. A new synthetic plastic for use in surgery. *J.A.M.A.* 135: 82–87, 1947.
- <sup>11</sup> JAKES, L. B. AND WATERS, E. T. Anaphylactic shock and heparin. *J. Physiol.* 99: 454, 1941.
- <sup>12</sup> JORPES, J. E. Heparin in the Treatment of Thrombosis, ed. 2. London: Oxford University Press, 1946.
- <sup>13</sup> LAMPERT, H. Die physikalische Seite des Blutgerinnungs Problems. Leipzig: Georg Thieme, 1931.
- <sup>14</sup> LOZNER, E. L. AND TAYLOR, F. H. L. Foreign surfaces and blood coagulation. *J. Clin. Investigation* 21: 241, 1942.
- <sup>15</sup> NIGGAARD, K. K. Hemorrhagic Diseases. Photo-Electric Study of Blood Coagulability. St. Louis: C. V. Mosby Co., 1941.
- <sup>16</sup> QUICK, A. J. The Hemorrhagic Diseases and the Physiology of Hemostasis. Springfield, Charles C. Thomas, 1942.
- <sup>17</sup> TOCANTINS, L. M. Relation of contracting surface and anticephalin activity to maintenance of fluidity and coagulability of blood. *Blood* 1: 156, 1946.
- <sup>18</sup> WARNER, E. D., BRINKOW, K. M. AND SMITH, H. P. The prothrombin conversion rate in various species. *Proc. Soc. Exper. Biol. & Med.* 41: 197, 1939.

# THE ROLE OF STAPHYLOCOAGULASE IN BLOOD COAGULATION

## II COAGULATION IN THE ABSENCE OF CALCIUM AND IN THE PRESENCE OF FLUORIDES, HEPARIN, AND AZO DYES

By JOHN B MIALT, M D

IN A preceding report<sup>6</sup> it was shown that broth cultures of staphylococci produce a substance (*staphylocoagulase*) which is able to clot plasma by reacting with a globulin substance (*coagulase globulin* CG) to form a thrombin-like substance (*coagulase-thrombin*, CT). Staphylocoagulase is not capable of clotting highly purified fibrinogen unless this reaction takes place, and the rate of production of CT when plotted gives a hyperbolic curve similar to that obtained in the activation of classic prothrombin to thrombin.

This similarity in the two reactions, plus the finding that in both cases the final product is one which clots highly purified fibrinogen, naturally leads to investigations aimed at establishing whether the two reactions are the same, similar, or unrelated. This report presents studies of the reaction of Staphylocoagulase with CG to form CT as influenced by factors known to have an effect on the conversion of prothrombin to thrombin and on the activity of thrombin.

### I *The Role of Calcium in the Staphylocoagulase Reaction*

It has long been known that the presence of oxalates or citrates does not interfere with the coagulation of plasma by staphylococci.<sup>15-17</sup> However, in the absence of quantitative studies it has been impossible to state that the presence of calcium ions is not necessary for the staphylocoagulase reaction. Because of the important role of calcium in the conversion of prothrombin to thrombin, a study of this factor in the staphylocoagulase reaction has been undertaken.

The method used was that of progressive removal of calcium by controlled molar concentrations of oxalate. Blood (human) was drawn into a Silicone\* coated syringe, and all the apparatus used in this section was similarly coated. Plasma was obtained by centrifugation and then diluted 1:10 with physiologic saline. The calcium concentration of the various reagents was determined by the method of Clark and Collip.<sup>8</sup> This made possible the addition of sodium oxalate in increasing molar concentrations beginning with a molar oxalate concentration just equal to the determined molar calcium concentrations and increasing to five times the calculated calcium equivalent.

Typical results are shown in table 1. It is obvious that progressive removal of calcium in no way interferes with the coagulation of plasma by either staphylocoagulase or CT, even when the plasma is otherwise incoagulable by an excess of thromboplastin. It seems justified to conclude that the reaction of staphylo-

From the Laboratories of the Marshfield Clinic and St. Joseph's Hospital, Marshfield, Wisconsin.

This study was supported in part by a grant from the Marshfield Clinic Research Foundation.

\* General Electric Co. Dri-Film #9987



coagulase with CG does not need the presence of calcium ions, differing therefore in a very basic way from the activation of prothrombin. On the other hand, once CT is formed calcium is no longer required, a condition also applicable to the action of thrombin.

## II Coagulant Action of Staphylocoagulase and CT in the Presence of Soluble Fluorides

The anticoagulant action of the soluble fluorides is due to their decalcifying action plus the apparent adsorption of prothrombin.<sup>2</sup> Table 2 shows that sodium fluoride even in very high concentrations has no effect on the coagulation of plasma by either staphylocoagulase or CT. The failure of fluorated plasma to clot on addition of thromboplastin plus adequate amounts of calcium chloride supports the opinion that fluorides also remove a substance necessary for coagulation, and this is substantiated by the more marked deficiency of clotting on simple recalcification.

TABLE 1—The Effect of Progressive Removal of Calcium on the Coagulation of Plasma by Staphylocoagulase and by CT

Molar Ratio Ca Oxalate	Clotting time with Staphylocoagulase <sup>1</sup>	Clotting time with CT <sup>2</sup>	Thrombin Time <sup>3</sup>	Clotting time with Thromboplastin <sup>4</sup>
1 0	45'	10'	15 3"	45"
1 1 0	45'	10'	15 2"	360"
1 2 0	45'	10'	15 4"	no clot
1 3 0	45'	10'	15 3"	no clot
1 4 0	45	10'	15 1"	no clot
1 5 0	45	10'	15 1"	no clot

Plasma (Silicone) 1 10 with sodium oxalate added to the given molar concentration final volume 0.5 cc. Incubated at 37 C for 10 minutes before adding other reagents then 0.5 cc. of coagulant added. Maintained at 37 C throughout.

<sup>1</sup> Staphylocoagulase, sterile cell free filtrate of broth culture 50 units<sup>20</sup>

<sup>2</sup> Coagulase Thrombin CT crude.<sup>20</sup>

<sup>3</sup> Thrombin (Upjohn) diluted with saline

<sup>4</sup> Thromboplastin (Difco).

It is noteworthy, therefore, that the effect of staphylocoagulase is not inhibited by this deficiency. If fresh oxalated plasma is treated with a suspension of CaF<sub>2</sub> it can thereafter not be clotted either by recalcification or by recalcification with an excess of thromboplastin added, suggesting that prothrombin is the substance removed.

## III Coagulant Action of Staphylocoagulase and CT in the Presence of Heparin

The anticoagulant action of heparin has been thoroughly investigated,<sup>4, 6</sup> and its use in this study is indicated by its inhibitory effect on prothrombin activation and its antithrombotic action in the presence of an accessory plasma factor. Therefore it seemed important to determine whether it inhibits the staphylocoagulase reaction or the action of CT.

Table 3 shows the results of various coagulation tests on plasma containing increasing amounts of heparin. No inhibitory effect on staphylocoagulase can be

TABLE 2.—*Constant Action of Staphylocoagulase and CT in the Presence of Sodium Fluoride*

Mol. Conc. of Fluoride <sup>1</sup>	Clotting Time with Staphylocoagulase <sup>2</sup>	Clotting Time with CT <sup>3</sup>	Thrombin Time <sup>4</sup>	Prothrombin Time <sup>5</sup>	Calcium Time <sup>6</sup>
0	30	10	19 0"	59 4"	9 0'
0 01 M	25	9	20 0"	55 1	15' 30"
0 02 M	25	9	21 0"	81 4"	no clot
0 03 M	25	9	20 5"	330 0"	00 clot
0 05 M	25	9	22 0"	00 clot	00 clot
0 10 M	25	9	19 5"	00 clot	00 clot
0 20 M	25	9	20 5"	no clot	00 clot
0 40 M	25	9	19 0"	00 clot	00 clot
1 0 M	25	9	25 0"	no clot	00 clot

<sup>1</sup> Oxalated human plasma (from 4.5 cc. of blood + 0.5 cc. of 0.1 M Na Oxalate) diluted 1:10 with saline 0.5 cc. containing the stated molar concentration of fluoride (from NaF) incubated for 10 minutes at 37 C before adding other reagents. All tests at 37 C.

<sup>2</sup> Staphylocoagulase, 50 units<sup>20</sup> 0.5 cc.

<sup>3</sup> CT Coagulase thrombin crude<sup>20</sup> 0.5 cc.

<sup>4</sup> Thrombio (Upjohn) diluted with saline

<sup>5</sup> Plasma 0.1 cc + Thromboplastin (Difco) 0.1 + CaCl<sub>2</sub> 0.1 cc of molarity calculated to equal the sum of oxalate plus fluoride in each test.

<sup>6</sup> Plasma 0.5 cc + CaCl<sub>2</sub> 0.5 cc. of molarity calculated to equal the sum of oxalate plus fluoride in each test.

TABLE 3.—*Effect of Heparin on the Clotting of Plasma by Staphylocoagulase and CT*

Heparin mg./cc. <sup>1</sup>	Clotting Time with Staphylocoagulase <sup>2</sup>	Clotting Time with CT <sup>3</sup>	Thrombin Time <sup>4</sup>	Prothrombin Time <sup>5</sup>	Calcium Time <sup>6</sup>
0	30'	10	15 6"	56 0"	5
0 005	30'	10	18 2"	86 4"	no clot
0 02	30	10'	19 3"	3600"	no clot
0 1	30	10	22 3"	00 clot	00 clot
0 2	30	10	32 1"	00 clot	00 clot
0 4	30'	10'	36 6"	00 clot	00 clot
0 6	30	10'	39 5"	00 clot	no clot
1 0	30'	10	65 9"	00 clot	no clot
2 0	30'	10	96 0"	00 clot	00 clot
5 0	30'	10	110 0"	00 clot	no clot

<sup>1</sup> Oxalated human plasma (from 4.5 cc. of blood + 0.5 cc. 0.1 M Na Oxalate) diluted 1:10 with saline 0.5 cc. containing the stated amounts of heparin (Heparin Sodium) incubated 10 minutes at 37 C before adding other reagents. All tests at 37 C.

<sup>2</sup> Staphylocoagulase, 50 units<sup>20</sup> 0.5 cc.

<sup>3</sup> CT Coagulase Thrombio crude<sup>20</sup> 0.5 cc.

<sup>4</sup> Thrombio (Upjohn) diluted with saline

<sup>5</sup> Plasma 0.1 cc + Thromboplastin (Difco) 0.1 cc + CaCl<sub>2</sub> 0.02 M, 0.1 cc

<sup>6</sup> Plasma 0.5 cc. + CaCl<sub>2</sub> 0.02 M, 0.5 cc

demonstrated, nor is there any evidence of inhibition of the CT effect. At the same time a definite inhibition of prothrombin activation is demonstrated, as well as a

clear cut antithrombic action, less striking in this case because of the use of diluted plasma for the experiments outlined

#### IV Coagulant Action of Staphylocoagulase and CT in the Presence of Azo Dyes

The anticoagulant action of certain azo dyes *in vivo* and *in vitro*<sup>16, 4</sup> has been attributed to the inhibition of thromboplastin<sup>16a, 17, 18</sup> or of thrombin.<sup>16</sup> Table 4 illustrates the anticoagulant effects of one of these dyes, Chlorazol Fast Pink, on plasma and also shows that there is no inhibition of either staphylocoagulase or CT. The anticoagulant action on plasma is very similar to that of heparin. In addition, the type of clots obtained with thrombin on plasma containing 2.0

TABLE 4.—Effect of Chlorazol Fast Pink on the Clotting of Plasma by Staphylocoagulase and CT

Dye Conc mg /cc <sup>1</sup>	Clotting Time with Staphylo- coagulase <sup>2</sup>	Clotting Time with CT <sup>3</sup>	Thrombin Time <sup>4</sup>	Prothrombin Time <sup>5</sup>	Calcium Time <sup>6</sup>
0	30'	10'	14 8"	52 0"	5'
0.01	30'	10'	15 0"	58 3"	8'
0.02	30'	10'	14 3"	56 1"	8'
0.03	30'	10'	14 3"	57 4"	8'
0.04	30'	10'	13 9"	73 7"	9'
0.05	30'	10'	11 5"	81 3"	13'
0.1	30	10'	17 4"	178 5"	no clot
0.2	30'	10'	20 0"	no clot	no clot
0.5	30'	10'	55 0"	no clot	no clot
1.0	30'	10'	120 0"	no clot	no clot
2.0	30	10'	"	no clot	no clot
5.0	30'	10'	"	no clot	no clot

<sup>1</sup> Oxalated human plasma (from 4.5 cc. of blood + 0.5 cc. 0.1 M Na oxalate), diluted 1:10 with saline, 0.5 cc. containing the stated concentration of Chlorazol Fast Pink (National Aniline, C.I. #353) incubated 10 minutes at 37°C before adding other reagents. All tests at 37°C.

<sup>2</sup> Staphylocoagulase 50 units<sup>22</sup> 0.5 cc.

<sup>3</sup> CT, coagulase thrombin crude,<sup>23</sup> 0.5 cc.

<sup>4</sup> Thrombin (Upjohn) diluted with saline.

<sup>5</sup> Plasma 0.1 cc. + Thromboplastin (Difco) 0.1 cc. + CaCl<sub>2</sub> 0.02 M, 0.1 cc.

<sup>6</sup> Plasma 0.5 cc. + CaCl<sub>2</sub> 0.02 M, 0.5 cc.

<sup>\*</sup> Extremely small globular or shredded clot after long incubation

and 5.0 mg/cc of dye suggests that there may be some stabilizing effect on fibrinogen as well, an effect attributed to the chemically related compound Germanin<sup>19, 25</sup>. It is interesting that the anticoagulant azo dyes, heparin, and Germanin contain ester sulfuric groups.

#### DISCUSSION

Following the demonstration by Arthus and Pagès<sup>1</sup> that the removal of calcium prevented the coagulation of blood, and the work of Pekelharing<sup>21, 22</sup> and Hammarsten<sup>18</sup> showing that it is essential for the formation of thrombin but not for thrombin activity, calcium has occupied a secure place in the classic scheme of blood coagulation.<sup>11, 23</sup> It is generally agreed that it is necessary for the activation

of prothrombin, probably through the formation of an intermediary complex<sup>12</sup>. Therefore since staphylocoagulase is active in the absence of calcium, we may suppose that its reaction with plasma does not involve the activation of plasma prothrombin. This conclusion is supported by the failure of heparin to inhibit the reaction in spite of its otherwise strong antiprothrombic effect, as well as the unreduced activity of staphylocoagulase in the presence of the other anticoagulants.

The failure of heparin and the azo dyes to inhibit the coagulation of plasma by CT suggests that the substance resulting from the reaction of staphylocoagulase and CG is not thrombin although it can be classified as thrombin-like. The nature of CT is not yet apparent, although it seems proper tentatively to place it in the group of substances which attack fibrinogen directly without the intervention of classic prothrombin and thrombin. Since we have consistently failed to demonstrate any fibrinolytic or fibrinogenolytic activity of the materials in the staphylocoagulase reaction it would appear justified tentatively to exclude it from the tryptic enzyme class. The inhibitory effect of heparin on tryptases<sup>14</sup> but not on staphylocoagulase would also support this tentative conclusion, as would the failure of fluorides to inhibit, the relatively wide pH range of activity, and other properties.<sup>20</sup> Obviously this and other points need further clarification. It is significant however that none of the other substances which are able to clot fibrinogen<sup>6, 7, 9, 10, 12</sup> have so far been demonstrated to need a globulin co-factor of the type involved in the staphylocoagulase reaction.

### CONCLUSIONS

1. The coagulation of plasma by staphylocoagulase and by CT does not require the presence of calcium.
2. The activity of staphylocoagulase and CT is not inhibited by soluble fluorides, heparin, or azo dyes.
3. The failure of antiprothrombic and antithrombic agents to inhibit the staphylocoagulase reaction indicates that it is not related to the activity of prothrombin.

### REFERENCES

- <sup>1</sup> ARTHUR M. AND PAGES, C. Nouvelle théorie chimique de la coagulation du sang. *Arch. de physiol. norm. et path.* Ser. 5, 2, 739, 1890.
- <sup>2</sup> BARRATT J. O. W. The action of anticoagulants. *J. Physiol.* 90, 377, 1937.
- <sup>3</sup> BORDET J. AND DELANOE L. Analyse et synthèse du processus de la coagulation. *Ann. et bull. Soc. roy. d. sc. méd. et nat. de Bruxelles* 72, 87, 1914.
- <sup>4</sup> BRAMBELL, T. W. R. AND PARKES, A. S. Effect of Chicago blue and chlorazol blue on clotting time of blood and on ovulation in rabbits. *J. Physiol.* 74, 65, 1932.
- <sup>5</sup> CHARGAFF E. The Coagulation of Blood. *Adv. in Enzymol.* 5, 31, 1945.
- <sup>6</sup> — AND BENDICH A. On coagulation of fibrinogen. *J. Biol. Chem.* 149, 93, 1943.
- <sup>7</sup> — AND COHEN S. S. Thromboplastic protein: structure, properties, disintegration. *J. Biol. Chem.* 156, 161, 1944.
- <sup>8</sup> CLARK, E. P. AND COLLIP J. B. A study of the Tisdall method for the determination of blood serum calcium with a suggested modification. *J. Biol. Chem.* 63, 461, 1925.
- <sup>9</sup> EAOLE, H. Coagulation of blood by snake venoms and its physiological significance. *J. Exper. Med.* 65, 613, 1937.

- <sup>10</sup> — AND HARRIS T N Studies in blood coagulation coagulation of blood by proteolytic enzymes (trypsin pepsin) *J Gen Physiol* 20 543 1937
- <sup>11</sup> FEROUSON J H The blood calcinm and the calcium factor in blood coagulation *Physiol Rev* 16 640 1936
- <sup>12</sup> — An intermediary calcium complex in blood coagulation *Am J Physiol* 119 755 1937
- <sup>13</sup> — AND ERICKSON B N Coagulant action of crystalline trypsin cephalin and lung extracts *Am J Physiol* 126 661 1939
- <sup>14</sup> GLAZKO A J AND FERGUSON J H Inhibition of tryptases by heparin *Proc Soc Exper Biol. & Med* 45 43 1940
- <sup>15</sup> GRATIA A De la coagulation du plasma oxalate par le staphylocoque *Compt rend Soc de biol* 82. 1393, 1919
- <sup>16</sup> HAMMARSTEN O Über Bedeutung der löslichen Kalksalze für die Faserstoffgerinnung *Ztschr f physiol Chem* 22 333 1896
- <sup>16a</sup> HUGGETT A Sr G The mechanism of the anticoagulant action of azo dyes on blood clotting *Quart J Pharm & Pharmacol* 1 372 1934
- <sup>17</sup> — AND ROWE F M The relationship of azo dyes to the coagulation of blood *J Physiol* 80 82, 1933
- <sup>18</sup> — AND SILMAN H The anticoagulant action of chlorazol *J Physiol* 74. 9p 1932.
- <sup>19</sup> LENGGENHAGER K. Ittwege der Blutgerinnungsforreshung *Klin Wehnschr* 15 1835 1936
- <sup>20</sup> MIALE J B The role of staphylocoagulase in blood coagulation I The Reaction of staphylocoagulase with coagulase globulin (CG) to form coagulase thrombin (CT) *Blood* 4. 1039 1949
- <sup>21</sup> PEKELHARINO G A Über die Bedeutung der Kalksale für die Gerinnung des Blutes *Internat Beitrage z. wiss Med (Festschr R Virchow)* 1 433 1891
- <sup>22</sup> — Untersuchungen über das Fibrinferment *Amsterdam* 1892.
- <sup>23</sup> QUICK A J Calcium in the coagulation of the blood *Am J Physiol* 131 455 1940
- <sup>24</sup> — The Hemorrhagic Diseases and the Physiology of Hemostasis *Springfield Chas C Thomas* 1942.
- <sup>25</sup> — The relationship of the antithrombogens heparin and chloromine fast pink to the antithrombin normally occurring in blood *J Biol Chem* 119 81 1937
- <sup>26</sup> ROUS P GILDING A P AND SMITH F The gradient of vascular permeability *J Exper Med* 51 807 1930
- <sup>27</sup> Walston H D The clotting of plasma through staphylococci and their products *J Hyg (Cam bridge)* 35 549 1935

# THE DETERMINATION OF THE LEVEL OF LEUKOCYTES IN THE BLOOD STREAM WITH INFLAMMATION: A THERMOSTABLE COMPONENT CONCERNED IN THE MECHANISM OF LEUKOCYTOSIS

By VALY MENAÏN, M.D.

*With the technical assistance of RUTH LEWIN AND LOUISE PIROVANE*

WITH AN acute inflammation the level of white cells in the circulation is altered, but it is difficult to predict the direction of the shift. There either may be an increase or a decrease in the number of circulating leukocytes, on the other hand there may be no appreciable change. The rise is in part due to the liberation by cells, severely injured at the site of an acute inflammation, of a specific type of alpha globulins\* termed as an entity, the leukocytosis-promoting factor (LPF).<sup>1-3</sup> Subsequent work has revealed that the active principle responsible for the effect of the LPF seems to be a polypeptide.<sup>4</sup> The rise in the number of white blood cells accompanying an inflammation cannot always be duplicated in magnitude by a single injection of the LPF. It is true that the constant production of the factor at the site of an acute inflammation may in part explain the level rising at times far above that obtained by merely injecting one dose of the LPF. On the other hand, it is conceivable that there may be other factors involved to explain the ultimate effect on the leukocyte level accompanying an acute inflammation.

Earlier studies have revealed that subsequent to the injection of the whole cuglobulin of an exudate, there often develops a leukocytosis.<sup>5</sup> At the time, it was surmised that necrosin associated with the cuglobulin fraction of an usually acid exudate by inducing tissue injury, in turn causes the local release of the leukocytosis-promoting factor with the eventual effect noticed.<sup>5</sup> This proved, however, to be an incorrect interpretation, for when necrosin was eliminated from the cuglobulin fraction, the noninjurious pyrexin or the pyrogenic factor of exudates still displayed the subsequent leukocytosis.<sup>6</sup>

The purpose of the present paper is twofold. In the first place, it will be shown that besides the thermolabile LPF of exudates,<sup>1</sup> there is also present a thermostable component, especially in acid exudates, which aids in explaining the mechanism of the final leukocytosis with inflammation. In the second place, an attempt will be made to point out the various factors concerned in the determination of the white blood cell level when there is a concomitant acute inflammation. It will be shown that the final picture is a resultant of the effect of the various factors previously described,<sup>7</sup> as well as the effect of the present component.

From the Agnes Barr Chase Foundation for Cancer Research, Temple University School of Medicine, Philadelphia, Penna.

Aided in part by a grant from the National Advisory Cancer Council. This is number 41 of a series entitled "Studies on Inflammation."

\* At least it seems to be associated with these proteins.

## METHODS

Dogs were given an acute pleural inflammation either with turpentine or with 5 per cent croton oil in olive oil. The amounts injected in the right chest cavity were either 1 to 1.5 cc. of turpentine or in one instance 0.3 cc. of 5 per cent croton oil in olive oil. On the following day and on subsequent days white blood counts were taken. The animals were tapped for the presence of any pleural exudate. The pH of the exudative material was measured by means of a Beckman pH Meter. The measurements were also checked by the use of Hydrion paper as a colorimetric measurement of the pH. Five to 1 cc. or even less was injected into the heart of a normal animal in which the basal white blood count had already been established. The number of circulating leukocytes was measured at hourly intervals for a period ranging from four to six hours. Into another dog a similar experiment was run in like manner, the only difference being that the injected exudative sample was either brought to a boil or boiled for thirty minutes. In this way any heat stable factor present in the exudative material could be recovered and its activity on the number of circulating leukocytes established. Since the primary activity, as it will be pointed out presently, of a heat stable component of the leukocytosis promoting factor seems to be located in exudates of an acid pH, this suggested that the effect may be associated with pyrexin, as indicated in earlier studies.<sup>6</sup> For this reason pyrexin was administered to normal dogs. These animals were observed at periodic intervals and as stated previously they eventually developed a marked leukocytosis in the blood stream.<sup>6</sup> This rise in the number of circulating leukocytes was observed to occur both in the systemic and the peripheral circulations, so that the effect could not very well be explained as a circulatory redistribution of white cells. Animals were also injected with boiled pyrexin in order to determine the heat stability of the leukocytosis-promoting component studied. In both experiments a differential count was made at the same time the absolute white cell level was determined. Particular care was given to establishing the percentage of immature or one lobe form of granulocytes as early as one hour after the administration of either boiled exudate or of boiled pyrexin. The reason for this step was to obviate the possibility of secondary injury in tissue caused by either exudate or pyrexin with subsequent formation of the LPF.

Finally, in an effort to determine whether the results obtained were specific for one species only, a similar type of experiment with whole and boiled guinea pig exudate was repeated on guinea pigs. The injection of guinea pig serum was utilized as a control in this series of experiments. Differential leukocytic studies were also undertaken. This study on dogs and on guinea pigs allowed a greater latitude in drawing final conclusions.

## RESULTS

When an acute inflammatory process is induced in the right pleural cavity, exudative material is often recovered and the pH of the exudate tends to be at an alkaline pH.<sup>8</sup> At times, however, the pH of the exudate may reveal a relatively neutral pH. On subsequent days the pH tends to be relatively at an acid pH.<sup>8</sup> When the pH of the exudate is alkaline or relatively neutral, it is found that the corresponding leukocyte level in the blood stream is either markedly increased, not significantly changed, or merely slightly increased. In some cases there is even a decrease in the level of circulating leukocytes. Such a decrease is likely to occur if the basal white blood cell count tends to be elevated. In other words, it is impossible to predict with any sense of precision what the injection of an irritant in the pleural cavity will do to the white cell count in the blood stream. Such data is assembled in table 1. On subsequent days the exudate recovered from such an acutely inflamed area tends to be at an acid pH,<sup>8</sup> and, generally speaking, the level of circulating leukocytes tends to be at a high level. This is illustrated in table 2. The question which immediately is raised is whether there is any single factor or a combination of factors which determine the ultimate picture in the level of leukocytes in the circulation with a concomitant acute inflammation.

If one groups all the exudative samples recovered in accordance with their hydrogen ion concentration, it is found that the final results are somewhat different in nature. These data are collected in table 3. It is readily seen that the most effective exudates in inducing a rise in the white blood cell count in a period from four

TABLE 1.—*The Presence of an Alkaline or an Essentially Neutral Exudate in an Inflamed Area and the Number of Circulating Leukocytes*

Dog no	Approximate duration of inflammation	Basal white blood cell count before inducing inflammation	pH of exudate	White blood cell count with inflammation at an alkaline pH or at a relatively neutral pH
	days	per cu mm		per cu mm
110-T	1	9,350	7.0	21,400
114-T	1	15,100	†	36,500
120-T	1	12,850	7.3	21,900
120-T	2†	12,850	7.8	13,500
121-T	1	27,050	8.0	15,650
121-T	3	27,050	7.5	26,200
121-T	4	27,050	6.95	10,500
87-T	5	27,050	7.15	11,350
126-T	1	11,100	†	21,950
127-T	1	13,650	†	24,450
127-T	1	9,800	†	44,450
127-T	2†	9,800	7.45	15,700
130-T	3†	9,800	7.55	14,900
135-T	7	7,800	7.03	44,900
135-T	3	20,500	7.52	19,000
135-T	4	20,500	7.0	33,100
135-T	5	20,500	6.98	25,850
132-T*	3	14,500	7.6	27,600

\* 5% croton oil in olive oil used as an irritant in all others turpentine utilized.

† Rejected

‡ No exudate obtained

TABLE 2.—*The Presence of an Acid Exudate in an Inflamed Area and the Number of Circulating Leukocytes*

Dog no	Duration of inflammation	Basal white blood cell count before inducing inflammation	pH of exudate	White blood cell count with inflammation at an acid pH
	days	per cu mm		per cu mm
110-T	2	9,350	6.5	35,250
87-T	4	11,100	6.7	9,050
130-T	3	7,800	6.3	55,550
130-T	4	7,800	6.87	55,150
130-T	5	7,800	6.8	28,450

to six hours are at a neutral pH. The average increase is 65.3 per cent. At an alkaline pH the increment averages 38.9 per cent, whereas at an acid pH the value of all the observations show a rise of 56.6 per cent.

If the samples of exudate are now subjected to heat by boiling them either for



thirty minutes or by bringing them just to a boil, it is found that the heat essentially abolishes the effectiveness of alkaline exudates. The average increase is 25.6 per cent (table 4). This increment is within the range of normal variation<sup>1</sup> during a

TABLE 3.—*The Effect of Inflammatory Exudates of Different Hydrogen Ion Concentration on the Number of Circulating Leukocytes*

Dog no	pH of injected exudate	Amount of exudate injected	Basal white blood cell count	Highest white blood cell count subsequent to the administration of exudate
		cc	per cu mm	
118 T	7.3	1.0	9,750	15,450
119 T	7.8	1.0	17,175	20,900
122 T	8.5	3.25	5,725	9,150
98 T	7.15	2.5	11,650	13,150
129 T	7.45	5.0	12,050	19,050
118 T	7.55	2.5	14,525	21,950
128 T	7.52	5.0	11,475	21,100
132 T*	7.6	5.0	19,325	20,455
Average change in white count at alkaline pH			12,709	17,651
87 T	7.05	6.0	9,100	12,250
63 T	7.0	10.0	12,475	19,600
122 T	±7.0	5.0	7,075	17,500
137 T	7.0	3.5	12,183	14,150
98 T	6.98	3.8	9,200	21,550
137 T*	6.95	5.0	14,575	18,300
122 T	6.95	3.5	6,975	15,000
Average change in white count at essentially neutral pH			10,216	16,907
87 T	5.0	5.0	10,700	24,200
98 T	6.5	4.0	10,500	11,750
119 T	6.2	5.0	14,900	20,500
131 T	6.63	2.0	12,250	10,950
132 T	6.87	5.0	11,425	24,100
133 T	6.8	5.0	17,650	33,750
Average change in white count at acid pH			11,204	17,542

Per cent increase in white cells with injection of alkaline exudate 38.9%.

Per cent increase in white cells with injection of neutral exudate 65.3%.

Per cent increase in white cells with injection of acid exudate 56.6%

\* 5% croton oil in olive oil used as an inflammatory irritant in all other experiments turpentine utilized

period of four to six hours. Heat at such temperature or even at a lower temperature evidently inactivates the LPF present in the exudate.<sup>1</sup> This is also essentially true when the original exudate is at a relatively neutral pH, the rise averaging 31.8 per cent (table 4). On the other hand, when the pH of the exudate is definitely acid in

character, the effectiveness of the active principle tends to be maintained (table 4). The average rise in this group of dogs is 55.5 per cent. This figure is approximately

TABLE 4.—The Effect of Boiled Exudates of Different Hydrogen Ion Concentration on the Number of Circulating Leukocytes

Dog no.	pH of injected exudate	Amount of boiled exudate injected	Basal white blood cell count	Highest white blood cell count subsequent to the administration of boiled exudate
		"	per cu mm	
119 T	7.3	1.0	16,800	19,050
118 T	7.9	1.0	8,175	9,850
123 T	8.5	3.25	14,625	19,900
124 T	7.15	2.5	14,250	14,150
125 T	7.45	5.0	7,550	12,750
149 T	7.55	2.5	14,225	14,500
129 T	7.5-	5.0	9,700	18,450
135 T†	7.6	5.0	12,700	14,450
Average change in white count at alkaline pH			12,253	15,388
94 T	7.05	6.0	14,475	16,500
54 T	7.0	11.0	13,600	19,050
123 T	±7.0	5.0	16,150	16,500
136-T*	7.0	3.5	14,783	21,275
118 T*	6.98	3.8	12,100	17,900
140-T†	6.95	5.0	9,950	15,000
87 T	6.95	3.5	10,000	13,800
Average change in white count at essentially neutral pH			13,008	17,146
80-T	5.0	0.7	9,250	19,250
97 T	6.5	4.0	8,900	10,500
118 T	6.2	1.0	12,775	19,950
132 T	6.63	2.0	13,200	16,300
131-T	6.87	5.0	11,700	16,450
134 T	6.8	5.0	15,475	28,450
Average change in white count at acid pH			11,883	18,483
Per cent increase in white cells with injection of boiled alkaline exudate			25.6%	
Per cent increase in white cells with injection of boiled neutral exudate			31.8%	
Per cent increase in white cells with injection of boiled acid exudate			55.5%	

\* In these experiments the exudate was boiled for thirty minutes in all other experiments the exudate was just brought to a boil.

† 5% croton oil in olive oil used as an inflammatory irritant in all other experiments turpentine utilized.

similar to the one encountered in case of unheated acid exudate (cf. table 3 and table 4). It would seem as if there is present a thermostable factor in acid exudates which tends to be either absent or present in reduced amounts in alkaline or neutral

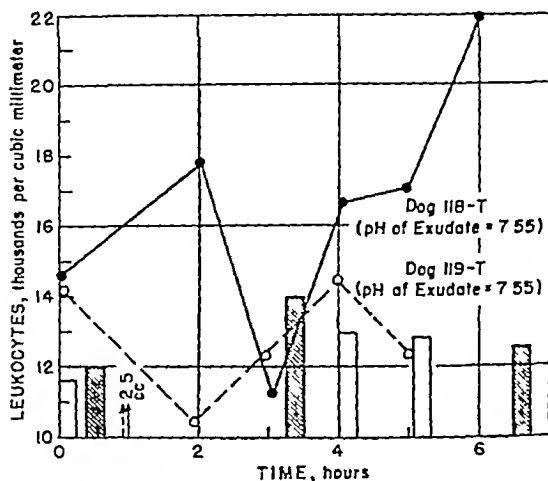


FIG. 1.—THE EFFECT OF AN ALKALINE EXUDATE ON THE NUMBER OF CIRCULATING LEUKOCYTES IN AN OTHERWISE NORMAL DOG —●— The effect of an exudate of pH 7.55 ○---○--- The effect of boiling this same exudate. Note that the effect on the absolute white count is abolished but that there is still an effect on the percentage of discharged immature granulocytes in the circulation. This is indicated by the unstained columns, whereas the black columns indicate the effect of an untreated alkaline exudate on the percentage of immature granulocytes in the circulation. Boiling the exudate does not seem to alter materially any change in the outpouring of immature granulocytes into the blood stream.

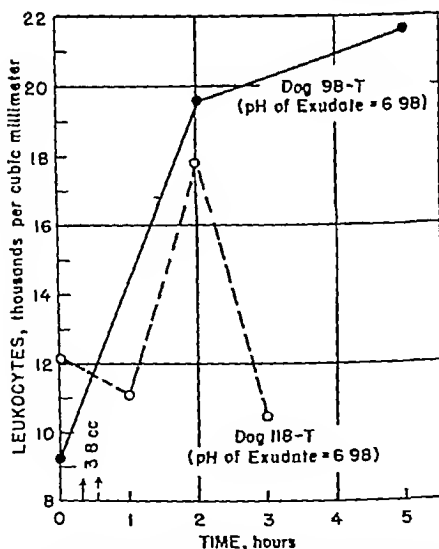


FIG. 2.—THE EFFECT OF AN EXUDATE AT A RELATIVE NEUTRAL pH ON THE NUMBER OF CIRCULATING LEUKOCYTES —●— The effect of an exudate at pH 6.98 ○---○---○ The effect of boiling the same exudate. Note that boiling an essentially neutral exudate does not completely abolish the effect on the absolute white blood count.

exudates. Extending the period of boiling the exudate thirty minutes makes no appreciable difference in the end results obtained. Croton oil in olive oil as an irritant yields similar effects as are obtained with turpentine (table 4). The effect of whole exudate at various pH is represented in the case of single experiments in figures 1, 2, and 3. It is seen that as far as the absolute white count is concerned, boiling an alkaline exudate tends to inactivate it (fig. 1). On the contrary, boiling

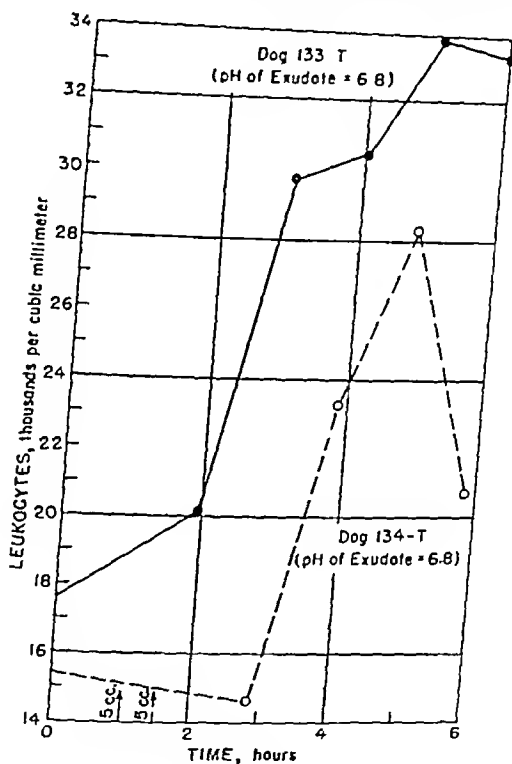


FIG. 3.—Boiling an acid exudate, although reducing the ultimate effect probably by abolishing whatever thermolabile LPF may be present in such an exudative material, still leaves a potent effect on the number of circulating leukocytes. —●— The effect on the circulating leukocytes of an exudate at pH 6.8. ○—○ The effect of a similar sample of exudate but brought to a boil before administration.

an acid exudate, although perhaps it diminishes its potency, still does not interfere with its effectiveness (cf. figs. 1 and 3). At relatively neutral pH, boiling the exudate yields somewhat of an intermediate picture (fig. 2).

As pointed out previously, pyrexin or the pyrogenic factor of exudate, the existence of which has been confirmed by Bennett,<sup>9</sup> tends to be present in acid exudates.<sup>10</sup> Smith and Smith have failed to obtain it by utilizing the scheme of extraction outlined by this author, but they have nevertheless shown its presence in the

euglobulin fraction of exudates <sup>11</sup> Tanturi and his collaborators have also shown that there is a pyrogenic factor present in the whole euglobulin fraction of exudates, or as it was formerly called necrosin <sup>13</sup> The term necrosin referred to a combination of necrosin, pyrexin, and the leukopenic factor of exudates Since that time these various components have been dissociated from the euglobulin fraction, particularly of acid exudates <sup>6 14 15</sup> At times the dissociation of pyrexin from necrosin is a difficult procedure It is assumed that this is the difficulty encountered by Smith and Smith <sup>11</sup> In recently published data, the author has shown that when such separation is difficult to perform, allowing necrosin to stand on ice for several weeks will permit pyrexin to settle at the bottom of the container as a precipitate which can easily be recovered as the pyrogenic factor, or pyrexin <sup>15a</sup>

TABLE 5—*The Eventual Effect of Pyrexin on the Number of Circulating Leukocytes*

Dog no	Basal white cell count	Dose of pyrexin	Highest white cell count subsequent to the administration of pyrexin
	per cu mm		per cu mm
97 T	12,350	36	17,700
70-T	12,700	7	20,950
18 D	11,400	±40	17,~50
26 D	9,775	24	16,950†
26-D	11,900	24	22,400‡
52 D*	20,800	89	33,100
75 D	11,250	134	17,400
81 D	8,925	20	15,750
12 D	8,500	23	24,750
8 D	10,250	45	20,250
Average	11,785		20,650

\* Pyrexin injected into right chest instead of introducing it in the circulating blood

† Peripheral blood sample

‡ Cardiac blood sample.

It has been pointed out previously that pyrexin, usually obtained from acid exudates, <sup>10</sup> most frequently in the later stages after its administration tends to yield a leukocytosis in the blood stream <sup>6</sup> Such observations are gathered in table 5 The factor which induces a state of leukocytosis and which is associated with pyrexin probably is closely related, if not identical, to the leukocytosis-promoting factor of acid exudates (tables 3 and 4), for boiling pyrexin fails to inactivate this leukocytosis component These data are assembled in table 6 Vigorous boiling fails to inactivate the leukocytosis-promoting component associated with pyrexin This indicates that this heat stable component seems to differ from the thermolabile LPF <sup>1</sup> When pyrexin or boiled pyrexin is administered, the state of leukocytosis may take place in a few hours or it may occur on the next day The boiled exudate, or for that matter boiled pyrexin, induces a discharge of immature or 1-lobe forms of granulocytes into the circulation as early as one hour, if not before, after the administration of the material These studies appear in table 7 The rapidity of effect

TABLE 6—The Effect of Boiled Pyrexin on the Number of Circulating Leukocytes

Dog no.	Total white cell count	Dose of boiled pyrexin	Highest white cell count subsequent to the administration of boiled pyrexin
	per cu mm	mg	per cu mm
97 T	12,325	±70	21,050
100 T	12,225	36	19,000
63 T	8,700	±7	13,750
100 T	9,225	±7	22,050
99 T	7,400	±250	3,350
97 T	12,075	98	23,950
119 T	11,175	±200	20,750
119 T	12,000	80	11,500
12 D	11,100	24	31,200
Average	10,825		20,733

TABLE 7—The Effect of Boiled Exudate or of Pyrexin on the Immature Granulocytes in the Blood Stream

Dog no.	Type of amount of boiled material injected	pH of exudate	Basal number of immature granulocytes (1-lobe form)	Number of immature granulocytes approximately 1 hour after administration of material (1 lobe form)	Number of immature granulocytes at the peak of the eventual leukocytosis after administration of boiled material (1 lobe form)
	exudate cc		per cent	per cent	per cent
119-T	2.5	7.55	16	32	30
136-T*	3.5	7.0	10	30	46
118-T*	3.8	6.98	16	26	32
140-T†	5.0	6.95	4	18	40
134 T	5.0	6.80	10	34	44
132 T	2.0	6.63	14	26	36
118 T	1.0	6.20	12	32	48
80-T	0.7	5.0	8	32	54
	pyrexin, mg				
118 T	±80	—	16	20	45‡
63 T	±7	—	6	40	46
80-T	±7	—	4	18	38
Average			11	28	42

\* The exudates sample was boiled for a period of thirty minutes in all other experiments the exudate was just brought to a boil

† 5% croton oil in olive oil used as a pleural irritant in all other experiments turpentine utilized

‡ This animal never displayed a leukocytosis after the injection of boiled pyrexin yet the number of immature granulocytes began rising as early as one hour after injection of the boiled pyrexin preparation

would support the view that the effectiveness of the heat stable component does not seem referable to a secondary tissue injury caused by either boiled exudate or boiled pyrexin

Furthermore, it is of interest that even though boiling tends to inactivate an alkaline exudate when the absolute white cells are studied, yet the percentage of immature granulocytes or single lobed forms still show a rise (fig 1). This would suggest that a study of the differential leukocytic formula is a more delicate test

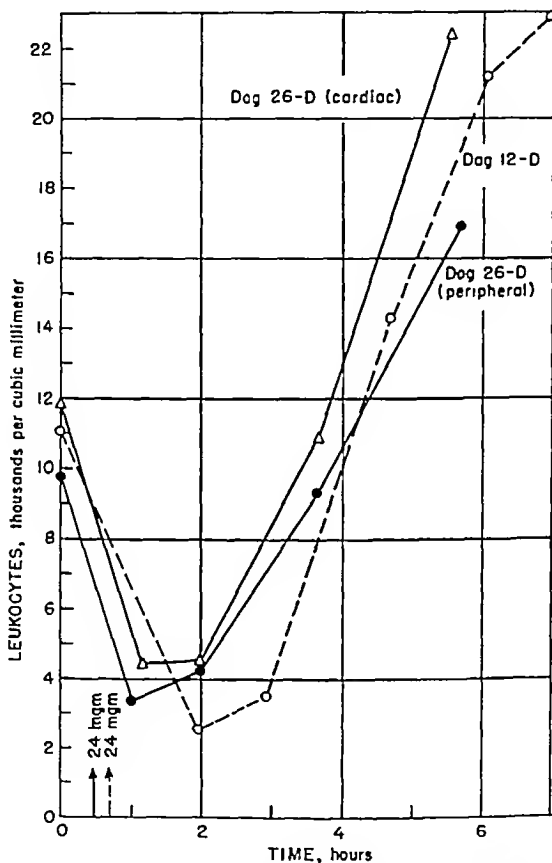


FIG 4—THE EFFECT OF PYREXIN AND OF BOILING THIS MATERIAL ON THE NUMBER OF CIRCULATING LEUKOCYTES —●— △—△—△ The effect of pyrexin on the number of circulating leukocytes ○—○—○ The effect of boiled pyrexin on the number of circulating leukocytes Note that either pyrexin or boiled pyrexin eventually gives rise to a leukocytosis The initial leukopenia has been shown in an earlier study to be referable to the presence of a leukopenic factor associated with pyrexin \* Samples of blood from the heart or from a peripheral vessel in the lobe of the ear produced essentially similar effects, indicating that the results are not referable to a redistribution of leukocytes in the circulation

than the absolute white count in determining the effectiveness of heated or unheated exudate That this is true has been pointed out previously in the case of rabbit LPF<sup>16</sup>

The effect of pyrexin and of boiled pyrexin is shown in the case of an experiment

in figure 7. It is seen that boiling the material, as in many cases of acid exudates, essentially fails to alter the ultimate effect of pyrexin in inducing a state of leukocytosis in the circulation (cf figs 3 and 4). Peripheral and cardiac samples of blood yield similar results, indicating that the effect is not referable to a redistribution of leukocytes in the vascular system (fig 4).

In order to avoid any criticism of drawing conclusions based only on one animal species, the foregoing series of experiments were repeated on guinea pigs. These

TABLE 8—*The Effect of Untreated and Heated Guinea Pig Exudate on the Circulating White Cells of the Guinea Pig*

G.P. no.	pH of exudate	Amount of heated or untreated exudate injected	Untreated Exudate		G.P. no.	Boiled Exudate	
			Basal number of circulating white cells	Highest number of circulating white cells within 6 hours		Basal number of circulating white cells	Highest number of circulating white cells
		cc	per cu mm	per cu mm		per cu mm	per cu mm
6	8.18	0.1	14,750	28,500	5	13,750	21,500
10	6.35	0.4	10,375	19,000	9	14,875	18,250
11	6.9	0.4	7,125	19,250	12	5,625	14,000
15	7.76	0.5	10,250	40,750	16*	11,125	29,000
Average			10,625	26,875		11,344	20,688

*The Effect of Untreated and Heated Guinea Pig Blood Serum on the White Blood Cell Level of the Guinea Pig*

20	—	0.5	15,000	19,000	22*	11,375	16,000
21	—	0.5	11,000	21,000	23*	6,875	8,750
Average			13,000	20,000		9,125	12,375

Average Maximal Increase in Circulating White Cells in a Study of Normal Variation in Guinea Pigs (within about 5 hrs) = 36.9%

(Taken from Proc. Soc. Exp. Biol. & Med. 61: 318-323, 1946).

Per cent increase with exudate 152.9%

Per cent increase with boiled exudate 82.4%

Per cent increase with blood serum 53.8%

Per cent increase with boiled blood serum 35.6%

\* When boiled there was hardly any coagulated exudate left. The material to be administered was therefore taken and suspended in about a two or three fold volume of distilled water.

latter animals were injected in the right pleural cavity with about 0.2 cc of 5 per cent croton oil in olive oil, and on the following day either the animal was found dead or else thoracentesis yielded a small amount of exudate ranging from 0.1 cc. to 0.5 cc. Postmortem examination revealed the presence of some exudative fluid in the right chest cavity, and this fluid was injected into normal pigs. They received 0.1 to 0.5 cc of either the untreated or the boiled material by injecting it subcutaneously in the groin. White blood cell counts were done by nicking the toe pads at approximately hourly intervals. It is clear from table 8 that exudative material, particularly at an alkaline pH, yields a pronounced rise in the number of



circulating leukocytes of an otherwise normal guinea pig upon administration of the material by the subcutaneous route. Boiling the exudate sample reduced somewhat the effect, but a pronounced influence still remained. In all the observations the exudate induced an average rise in the number of total circulating leukocytes amounting to 152.9 per cent. Boiling such exudative material reduced this figure to 82.4 per cent. This is still an increase, for in an earlier study it has been shown that

Table 9—The Effect of Both Unbeated and Heated Exudate and Blood Serum from Guinea Pigs on Their Circulating Immature Granulocytes

G P no.	Amount and type of material injected	Basal number of immature granulocytes (1 lobe form)	Number of immature granulocytes approximately 1 hour after administration of material (1 lobe form)	Number of immature granulocytes at the peak of the eventual leukocytosis after administration of material (1 lobe form)
	cc	per cent	per cent	per cent
11	0.4 cc whole exudate	4	13	18
15	0.5 cc whole exudate	2	20	22
Average		3	16.5	20
5	±0.1 cc boiled exudate	0	6	14*
9	0.4 cc boiled exudate	4	8	28*
12	0.4 cc boiled exudate	4	10	22
16	±0.5 cc boiled exudate†	0	6	10
Average		2	7.5	18.5
20	0.5 cc whole blood serum	0	0	2
21	0.5 cc whole blood serum	2	4	4
Average		1	2	3
23	0.5 cc boiled blood serum†	6	4	8*
22	0.5 cc boiled blood serum†	2	4	4
Average		4	4	6

\* Percentage of immature granulocytes taken 1 to a few hours after the height of the leukocyte level has been attained.

† Since a very slight amount of the material was obtained following its boiling the material was suspended in distilled water to a volume ranging to about two or three times the amount of boiled serum.

the average maximal variation in guinea pigs is 36.9 per cent.<sup>17</sup> As a further control, the effect of guinea pig blood serum was injected subcutaneously in the groin of otherwise normal animals, this was done in only two animals (table 8). The average increase in the number of circulating leukocytes was found to be definitely less than in the case of exudates. It amounted to 53.8 per cent (cf. with 152.9 per cent in the case of exudates, table 8). Furthermore, when the serum was brought to

a boil, the average rise amounted to 35.6 per cent, a figure not above that encountered in a study of the normal variation in guinea pigs.<sup>17</sup>

Finally, a study was undertaken in guinea pigs to determine the effect of exudates on the differential leukocytic formula. It is clear, as shown in table 9, that a guinea pig exudate induces an early discharge of immature leukocytes into the circulation. This rise is to some extent duplicated in the case of boiled exudates (table 9). On the other hand, both unheated and boiled blood serum fail to show any appreciable changes. In brief, it would appear from the results on the guinea pigs as if in these animals there is also present in their exudative material a relatively thermostable leukocytosis-promoting factor which is essentially absent in guinea pig serum. These results resemble the observations made above on canine exudates.

### DISCUSSION

The foregoing experiments indicate the presence of an additional component concerned with the mechanism of leukocytosis with inflammation. In contrast to the factor previously reported which was thermolabile,<sup>1</sup> the present factor is thermostable. The thermolabile component is found to be associated with the alpha globulins of exudates.<sup>2</sup> The active principle seems to be a polypeptide, perhaps attached to the globulin molecule.<sup>4</sup> When separated from the globulins by aging the material, it is found that the original thermolabile LPF component, now freed of its protein attachment, is also thermostable.<sup>4</sup> The thermostable component described in this paper is usually recovered in greater abundance from acid exudates, in contrast to the labile component, and furthermore this component is found in close association with the pyrogenic factor of exudates or pyrexin. Are the thermolabile component and the thermostable component of the LPF separate substances? This may be the case. On the other hand, it is conceivable that one is dealing with one and the same substance. The leukocytosis-promoting factor, as a polypeptide, seems to attach itself in an alkaline or in a neutral exudative medium to alpha globulins.<sup>2</sup> Consequently, boiling this material denatures the protein and the thermolabile LPF attached to a denatured protein is inactivated. It is also quite possible that in an acid exudate with an appreciable amount of pyrexin present,<sup>10</sup> the LPF slides over and becomes correspondingly attached to the pyrexin molecule. Since pyrexin is also thermostable,<sup>14</sup> and since the LPF polypeptide is likewise thermostable,<sup>4</sup> boiling the combined factors fails to inactivate it. Future studies, it is hoped, will settle this point.

Furthermore, with an acute inflammation, the resultant level of the number of circulating leukocytes cannot be predicted with any degree of certainty (table 1). When the exudate is acid in character, the level in the blood stream tends to be markedly raised (table 2). It would seem as if the rise in the number of leukocytes in the blood is referable to at least two factors, the thermolabile and the thermostable components of the leukocytosis-promoting factor. This tendency is counterbalanced by two other leukopenic factors, the thermolabile leukopenin of exudates<sup>18</sup> and the thermostable leukopenic factor of primarily acid exudates.<sup>4</sup> The combination of these various opposing factors influences the final leukocytic level in the circulation. When the leukocytosis-promoting factors dominate the

resultant effect is a rise in the white blood level. On the other hand, a predominance in the concentration of either, or of both, leukopenic factors results in no appreciable change in the level, or else in a frank leukopenia.

### CONCLUSIONS

There is present in inflammatory exudates, particularly in exudates having an acid hydrogen ion concentration, a thermostable leukocytosis-promoting component, which in conjunction with the previously described thermolabile leukocytosis-promoting factor aids in our understanding of the mechanism of leukocytosis with many inflammatory states. The thermostable component of the leukocytosis promoting factor is recovered in association with the pyrogenic factor or pyrexin. Whether it is essentially different chemically from the active principle present in the thermolabile factor is discussed and remains to be seen.

The resultant leukocyte level in the blood stream with a concomitant inflammation is the resultant of a multiplicity of factors. The rise in the number of circulating leukocytes with inflammation is induced by a combination of the thermolabile and the thermostable components of the leukocytosis-promoting factor of exudates, whereas the decrease in white blood count seems referable to both leukopenin and the leukopenic factor of inflammatory exudates. A predominance in the concentration of any one of these factors liberated by injured cells at the site of an acute inflammation will determine the final level of white cells in the circulation.

### REFERENCES

- <sup>1</sup> MENKIN V. Studies on inflammation. XVIII. On the mechanism of leukocytosis with inflammation. *Am J Path* 16 13 1940.
- Mechanism of leukocytosis with inflammation. The nature of the leukocytosis promoting factor in exudates. *Arch Path* 30 363 1940.
- <sup>2</sup> DILLON M. L. COOPER G. R. AND MENKIN V. Electrophoretic studies on the leukocytosis-promoting factor of exudates. *Proc Soc Exper Biol & Med* 65 187 1947.
- <sup>3</sup> MENKIN V. The active principle in the leukocytosis promoting factor of exudates. *Blood* 3 939 1948.
- Chemical basis of injury in inflammation. *Arch Path* 36 269 1943.
- <sup>4</sup> — Leukopenia and inflammation. The presence of a leukopenic factor in inflammatory exudates. *Arch Path* 41 50 1946.
- Modern concepts of inflammation. *Science* 105 538 1947.
- <sup>5</sup> — Studies on inflammation. X. The cytological picture of an inflammatory exudate in relation to its hydrogen ion concentration. *Am J Path* 10 193 1934.
- <sup>6</sup> BENNETT I. L. JR. Observations on the fever caused by bacterial pyrogens. II. A study of the relationship between the fevers caused by bacterial pyrogens and by the intravenous injection of the sterile exudates of acute inflammation. *J Exp Med* 88 279, 1948.
- <sup>7</sup> MENKIN V. Further studies on the mechanisms of injury and fever with inflammation. *Fed Proc.* 4 149 1945.
- <sup>8</sup> SMITH O. W. AND SMITH G. V. S. Evidence that menstrual toxin and canine necrosin are identical. *Proc Soc Exper Biol & Med* 59 116 1945.
- <sup>9</sup> TANTURI C. A. CANEPA J. F. AND BANFI, R. F. Estudios sobre inflamacion. IV. Necrosina. *Revista Medicina* 6 143 1945.
- <sup>10</sup> MENKIN V. On the mechanism of fever production with inflammation. *Proc Soc. Exper Biol & Med* 54 184 1943.
- <sup>11</sup> — Chemical basis of fever with inflammation. *Arch Path* 39 28 1945.

- "—— Chemical factors and their role in inflammation Arch Path 41 376 1946
- "—— The Biochemistry of Cell Injury as Manifested in Inflammation Proceedings of the 6th International Congress of Experimental Cytology Stockholm July 1947 (published in Stockholm 1949) Page 511
- "—— Recovery of the leukocyte promoting factor in exudates of rabbits Proc Soc Exper Biol & Med 61 448 1947
- "—— MARTINSON M D and ULLÉN F A convenient test animal for the detection of the leukocyte-promoting factor of exudates Proc Soc Exper Biol & Med 61 318 1946
- "—— Mechanisms of leukopenia with inflammation An additional leukopenic factor found in alkaline exudates Arch Path 46 145 1948

## EXPERIMENTAL THORACIC DUCT FISTULA

### OBSERVATIONS ON LYMPHOCYTE OUTPUT

B<sub>y</sub> WILLIAM W L GLENN, M D , FRANCIS X BAUER, M D , AND  
SAMUEL L CRESSON, M D

SINCE the adaptation of polyvinyl chloride and polyethylene to the making of cannulae, several investigators<sup>1</sup> \* have been able to produce thoracic duct fistulae in the dog which are free-flowing for as long as 8 days. Thus is provided, for the first time, a means for continuing observations on the lymphocyte output from the thoracic duct. The findings may have implications as to the production, circulation and fate of the lymphocyte.

It has been known for many years that large numbers of white cells, 98 per cent of them lymphocytes, are constantly passing into the blood stream from the thoracic duct. Yoffey<sup>2</sup> has found that a 10 kilogram dog discharges, on the average, about 200 million lymphocytes hourly into the blood stream through the thoracic duct. From this rate of lymphocyte output he estimated that in the dog there is a complete replacement of the blood lymphocytes twice daily. Sanders, Florey and Barnes<sup>4</sup> and Adams, Saunders and Lawrence<sup>5</sup> have recently determined that there is a similar rate of replacement of the blood lymphocytes in the cat. The former also estimated that in the rabbit the blood lymphocytes are replaced at least 5 times a day. It is important to note, however, that in all of these previous experiments, the thoracic duct fistulae have been created under a general anesthetic and have drained for a few hours only.

Investigators are not in agreement over the fate of the lymphocyte after it enters the blood stream. Yoffey and Drinker,<sup>6</sup> for example, on the basis of the number of lymphocytes in lymph prior to passage through a lymph node, maintained that only one lymphocyte in 32 that enters the blood from the thoracic duct eventually re-enters a peripheral lymphatic. They believed that to this small extent a recirculation of lymphocytes takes place. Sanders, Florey and Barnes<sup>4</sup> did not believe that any lymphocytes are recirculated through the lymphatics and assumed that all of the lymphocytes passing through the thoracic duct are newly formed cells. Ehrlich,<sup>7</sup> on the other hand, thought that many lymphocytes leaving the blood are recirculated through the lymphatics. Considering the several theories regarding the fate of lymphocytes, Ehrlich stated: "After having been formed in the lymphatic tissue, the lymphocyte passes through the lymphatics, and possibly, also through the veins of this tissue, into the blood stream, where it circulates for several hours. After this period some leave the blood through the mucous membrane of the gastrointestinal tract, others, probably the majority, return to the lymphatic tissue, and in the lymph nodes, also through the peripheral lymph vessels. It is likely that many of these lymphocytes go on and return to the blood stream, and in fact this cycle may be repeated several times."

From the Surgical Research Department, Jefferson Medical College, Philadelphia, Penna.

Adams, Sanders and Lawrence<sup>3</sup> believed that although the recirculation of lymphocytes can be demonstrated it is not of great significance when one considers the total lymphocyte replacement occurring via the thoracic duct. In discussing the effect of thoracic duct cannulation on the concentration of blood lymphocytes, these same investigators concluded that the real test of the effect of cannulation of the thoracic duct would be to continue the cannulation for much longer periods of time. This was not possible before the use of plastic cannulae owing to the rapid clotting of lymph in the glass cannulae used by previous investigators.

### METHOD

Using dogs under local anesthesia the thoracic duct was cannulated in the neck with a cannula made from polyethylene tubing. The right lymphatic duct was ligated and divided a week or more prior to the thoracic duct cannulation. The technic is described in detail in another paper. Studies on the output of lymphocytes through the thoracic duct fistulae were carried out on 7 dogs. One experiment where lymphocyte counts were made has been omitted due to frequent clotting of the lymph in the cannulae. Many red blood cells were present in the thoracic duct lymph of this animal. Total white cell counts and differential smears were made at about the same time each day on peripheral venous blood. At least 400 cells were counted in the differential blood smears. Because of the difficulty of distinguishing large lymphocytes from mononuclear cells in the peripheral blood smears, small lymphocytes only are recorded. Total white cell counts on the lymph were usually made several times daily when the fistulae were flowing. The number of counts varying between a single count on one day in one experiment to 54 counts on one day in another experiment. Calculation of the lymph flow was made simultaneously with many but not all of the lymphocyte counts. Where simultaneous lymph flow was not determined the counts were excluded from the results. As pointed out by Rous<sup>4</sup> and Drinker and Yoffey,<sup>5</sup> the ideal method for determining the lymphocyte output through the thoracic duct would be to collect the lymph over a period of time, adding an anticoagulant, and then to count the cells in the measured volumes. In these experiments the volume of lymph drained, usually over a ten to thirty minute period, was measured and the lymphocyte output for that period was calculated from a count taken during the period directly from the cannula. At least one differential smear on the lymph was made each day at the same time as the differential blood smear.

### RESULTS

The results of these experiments are recorded in table 1. It is evident that there was usually a fall in the blood concentration of small lymphocytes. This fall was frequently marked but at no time did it reach zero. The blood concentration of small lymphocytes reached the lowest point generally on the second or third day of drainage and thereafter either remained the same or increased gradually. Where counts on the blood were continued after the fistula had closed it was noted that the return of the lymphocyte concentration in the blood to preoperative levels took place slowly. In experiment 8 a splenectomy was performed several weeks before the fistula was made. There was no greater decrease in the number of small lymphocytes in the blood in this dog than in the other dogs where the spleen was left intact.

A study of the lymphocyte concentration of thoracic duct lymph shows that where there was adequate lymph drainage beyond the second or third day there was usually a drop in the calculated number of lymphocytes passing out through the thoracic duct per unit of time. Experiment 1 is of particular interest. On cannulation of the thoracic duct in this dog a profuse flow of lymph and a relatively low lymphocyte count was found. It was decided to make a deliberate attempt to obtain a maximum flow of lymph during the period of fistula drainage. Fluids were administered chiefly by mouth. During the last twenty-four hours of fistula drainage more than 6000 cubic centimeters of fluid were given. The maximum measured flow of lymph during a one hour period on this day was 368 cubic centimeters. Despite this very profuse flow of lymph there was only a moderate drop in the calculated number of lymphocytes passing through the thoracic duct per unit of time (table 1).

In several experiments sufficient data was available so that observation could be made on the relationship between the rate of flow through the thoracic duct and the lymph count per unit of time.

TABLE I—Results of Experiments

Experiment	Date	Hematocrit (%)	W B C (per cu mm)	Small lymphocytes (per cu mm)	No of specimens	Lymph flow (cc. per hr)	W B C. (per cu mm.)	Remarks
2	10/24	40 0	16,800	1050				Very acute. Fluids forced. Death occurred one day following closure of fistula vasomotor collapse.
	10/27	40 0	8 500	1466				
	11/13	35 5	10 750	118				
	11/17	36 5	11 800	885				
	Fistula open 11/17				2	96 0	2825	
	11/18	49 0	14 500	108	2	111 7	1950	
	11/19	49 0	19 300	707	5	172 5	2290	
	11/21	57 0	23,150	347	5	239 0	830	
	Fistula closed 11/22							
4	1/19		12 500	1-13				Quiescent.
	1/20	44 0	17 000	1581				
	Fistula open 1/20				2	96 0	8000	
	1/21	53 0	33 200	166	3	61 8	6500	
	1/22	53 0	45 200	678	5	90 0	5070	
	1/23	56 0	32 650	3-7	6	64 0	2750	
	1/24	51 5	43,200	1080	6	70 2	3011	
	Fistula closed 1/25							
	1/25	48 0	43 800	326				
	1/26	34 0	2- 850	712				
	1/27	34 0	12,350	617				
	1/31	35 5	30 000	1440				
5	8/7	32 0	20,350	1278				No attempt made to increase lymph flow by forcing fluids.
	8/18	35 0	20 850	1679				
	8/19	29 0	29,200	1564				
	Fistula open 8/19				1	66 0	4200	
	8/20	39 0	44,900	898	1	32 7	4550	
	8/21	37 0	43,000	645	1	28 8	6800	
	8/22	34 0	28,850	938	2	32 6	3600	
	8/23	43 5	33,400	1085	1	26 0	3050	
	8/24	43 5	35 400	1770	2	20 6	3450	
	8/25	38 0	38 075	857	2	22 0	2067	
	8/26	38 0	45,325	1246	2	51 4	1800	
	Fistula closed 8/26							
	8/27	38 0	55,200	138				
	8/28	30 0	82,450	824				
	8/30	31 0	45,850	802				
	9/7	26 0	44,550	1225				
6	7/17	25 5	18,300	823				No preoperative blood counts made. First blood count - hrs. after fistula produced. Occasional diarrheal stool
	Fistula open 7/17				8	135 2	3606	
	7/18	35 0	65,700	1114	8	31 6	4525	
	7/19	36 0	68 300	853	9	64 2	3450	
	Fistula closed 7/20							

TABLE 1.—Continued

Flow (cc/min)	Date	Hemat (%)	WBC (per cu mm)	Cal culat ed No. of lymph ocytes per cu mm	Lymph flow (cc/min)	WBC per cu mm	Remarks
7	7/23	35.5	25,700				
	7/26	31.0	25,450				
	7/27	31.5	22,500				Cannula accidentally re- moved 7/29
	Fistula open 7/27				6	20.4	3942
	7/28	33.0	25,500		11	30.0	3174
	Fistula closed 7/29						
8	7/30	35.0	27,400	1844			
	7/31	40.0	30,350	1745			
	8/1	35.0	34,000	2040			
	Fistula open 8/1				8	48.6	9578
	8/2	35.0	48,900		31	43.2	8007
	8/3	42.0	61,800	155	20	44.0	4748
	8/4	33.0	59,800	1076	10	57.6	3952
	Fistula closed 8/4						Sedative doses of pen- torhal used
	8/5		42,700				
	8/6	25.0	37,900	474			
	8/7		34,700	867			
	8/16		22,800	1596			

of lymph early and late in the period of fistula drainage. It appeared that for the first two to three days after the fistulae were established an increase in the flow of lymph as followed the administration of fluid was accompanied by an increase in the number of lymphocytes per cubic millimeter of lymph. After several days of free drainage, however, an increase in lymph flow usually resulted in a fall in the number of lymphocytes per cubic millimeter of lymph. It was observed that if the lymph flow were allowed to fall to a very low level after a period of profuse flow a concentration of the lymphocytes took place for a time but as the flow continued to drop the number of lymphocytes decreased also. An increase in the lymph flow under these conditions was accompanied by a prompt rise in the lymphocytes in the lymph. In table 2 excerpts from the protocol on experiment 8 illustrate these changes.

Smears for differential counts made on specimens of thoracic duct lymph did not reveal any apparent changes at first but after several days of drainage there was observed a fall in the percentage of small lymphocytes (table 3). Although the total white cell count in the lymph at the time of the differential smear was sometimes higher on the final day of fistula drainage than on the first day it can be seen from table 1 that an average of several counts always revealed a lower total white cell count on the final day of drainage. The counts recorded in table 3 were made in the early morning when the lymph flow had diminished with consequent concentration of the cellular elements.

### DISCUSSION

The creation of a thoracic duct fistula under local anesthesia in the dog which will drain continuously for a number of days is not difficult but the animal so prepared requires almost constant attention during the period of fistula drainage. In several of the experiments reported the animals were observed continuously over consecutive 24 hour periods. In the majority of experiments, however, observations were made over a 16 to 18 hour period daily as long as the fistulae remained open.



TABLE 2.—Experiment 3 Thoracic Duct Lymph

Day of fistula drainage	Time	Lymph volume (cc)	Time	W B C (Per C.C.ML.)
	12 46-1 16	16 0	12 47	9,900
	1 46-2 16	13 7	1 48	9,450
	2 46-3 16	7 8	2 50	9,800
	3 46-4 16	6 4	3 48	14,575
	4 46-5 16	2 4	4 47	11,500
	5 16 Small clot removed from cannula			
	5 46-6 16	3 6	5 47	4,800
	6 46-7 16	2 3	6 47	4,900
	7 16 Small clot removed from cannula			
	7 46-8 16	0 2	7 47	4,700
	8 16 511 cc. 5% glucose in 0.85% saline started I V at about 3 34 cc/minute			
	8 46-9 16	5 0	8 52	3,850
	9 46-10 16	21 0	9 47	12,150
	10 46-11 16	18 0	11 00	11,525
4	9 30-10 00	12 6	9 30	4,150
	10 00-10 30	9 7	10 05	5,300
	12 00-12 15	2 0	12 17	7,350
	12 15 500 cc 5% glucose in 0.85% saline given P O			
	12 45-1 03	42 0	12 46	3,100
	1 03-1 15	27 5	1 03	2,550

TABLE 3.—Cell Counts Thoracic Duct Lymph

Experiment	Date	Total W B C (per cu mm)	Lymphocytes (%)			Polymorpho-nuclears (%)	Total counted
			Small	Intermediate	Large		
2	11/17	2,650	89 2	7 7	2 7	0 4	220
	11/21	4,650	72 6	18 1	7 5	1 8	94
4	1/20	7,100	83 1	14 8	1 5	0 6	842
	1/24	5,570	64 3	12 6	15 4	7 7	636
5	8/19	8,400	88 7	10 4	0 9	0 0	518
	8/26	1,650	72 0	19 0	2 0	7 0	100
6	7/17	2,550	62 4	25 3	12 0	1 3	300
	7/19	6,000	50 4	25 6	18 0	6 0	300
8	8/1	4,900	70 0	24 8	5 2	0 0	994
	8/4	5,300	62 6	31 2	6 2	0 0	451

The problems encountered when using plastic cannulae are relatively minor and in marked contrast to the difficulties encountered when using cannulae made of glass or other nonclot preventing materials. Nevertheless, plastic cannulae become blocked occasionally. Factors that predispose to blockage must be carefully

avoided especially the dehydration of the animal and contamination of the mouth of the cannula with tissue fluid from the wound through which the cannula protrudes. Small clots in the cannula should be watched for and promptly removed. This can best be accomplished by twisting a fine wire in the substance of the clot near the mouth of the cannula. If the lymph contains large numbers of red cells, which it occasionally does, early blockage of the cannulae can be anticipated. The loss of electrolytes, fat and protein through a continuously draining thoracic duct fistula is of major importance. An animal so depleted would soon die unless some effort were made to replace these losses. An attempt was made to replace the sodium and chloride loss but no protein or fat was administered except for a small measured amount of the latter given for the purpose of a fat absorption study. A detailed account of the protein loss in these animals is given in another paper. The protein concentration in the lymph and blood declined steadily during the period of fistula drainage, a fall of 2 to 2.5 grams per cent total protein over a period of four to six days of fistula drainage was the rule. Where done, the albumen-globulin rates remained the same after depletion but only a few such determinations were made.

The drop in the concentration of blood lymphocytes after thoracic duct fistulae were produced is in accord with the findings of others who have worked with thoracic duct fistulae of short duration. Sanders, Florey and Barnes<sup>4</sup> and Adams, Sanders and Lawrence<sup>5</sup> showed that a similar fall in the concentration of lymphocytes in the blood occurred following operations where no lymph fistulae were produced. The evidence of Adams, Sanders and Lawrence is particularly convincing on this point. The cats used in their experiments were anesthetized with dial and urethane intraperitoneally. The effects on the blood lymphocyte levels of the anesthetic agents and their mode of administration should probably also be checked since the drop in the blood lymphocytes in our experiments followed cannulation of the thoracic duct under local anesthesia.

The finding of a decreased lymphocyte output after several days of continuous lymph drainage is of considerable interest. If there is an appreciable recirculation of lymphocytes the failure of many millions of these cells to reach the blood would after a time cause a fall in lymphocyte output through the thoracic duct. On the other hand, a decreased lymphocyte production, in response perhaps to the prevailing experimental conditions, would likewise cause a drop in lymphocyte output. We have presented no conclusive evidence one way or the other. It has been demonstrated by Rous<sup>8</sup> and by Drinker<sup>10</sup> that there are many lymphocytes in the lymph nodes which, under conditions of normal lymph flow, do not enter the efferent lymphatics. With an increased flow of lymph through the nodes, however, there is a sharp increase in the number of lymphocytes in the efferent lymphatics. Although this indicates that there is a surplus of lymphocytes in the nodes it gives no indication whether these cells were formed in the nodes or whether they arrived there after having first circulated through the blood.

In experiments of several days' duration there are many factors which may influence lymphocyte production. Starvation, known to have a depressant effect on lymphoid tissue, may have been a factor in these experiments. According to Jackson,<sup>11</sup> inanition results in a characteristic atrophy of lymphoid tissue with prompt

recovery accompanying refeeding. He states that lymphoid tissue is particularly sensitive to fat deficient diets. In our experiments no protein and a negligible amount of fat was given during the period of fistula drainage, although all dogs received glucose or sucrose from which fat may have been formed.<sup>12</sup> The amount of sugar given varied from the equivalent of 15 calories per kilograms per day in experiment 8, to 50 plus calories per kilogram per day in experiment 2. Some of the carbohydrate given was undoubtedly lost through the fistulae. During the period of fistula drainage there was a rapid and usually appreciable fall in the protein concentration of both serum and lymph in all of these experiments.\* Jolly<sup>13</sup> observed an appreciable loss of weight in the cervical and popliteal lymph nodes, as well as in other lymphoid structures, of two dogs starved for six and seven days. In two of our experiments, 2 and 4, one popliteal node was removed under local anesthesia at the time that the thoracic duct fistulae were produced and the opposite popliteal node was removed, under local anesthesia, just after the fistula closed. There was a loss of weight in the nodes removed after fistula drainage that averaged 213.5 milligrams for the 2 animals. This was compared with the average difference in weight of 42.5 milligrams, with a maximum difference of 90.5 milligrams between the two popliteal nodes in 8 control animals sacrificed for other reasons. Microscopic sections of the glands in the 2 fistula dogs did not show any characteristic changes in the nodes removed after fistula drainage. It was not possible to correlate the decrease in lymphocyte output through the thoracic duct with the histologic findings in the popliteal nodes.\*

An increase in the blood leukocyte level accompanying the production of thoracic duct fistulae has been observed by Adams et al.<sup>6</sup> and others. With regard to the lymphopenia that usually accompanies a blood leukocytosis,<sup>6</sup> it is interesting to note (table 1) that the rise in leukocytes preceded any significant fall in the lymphocyte output through the thoracic duct fistula.

The significance of the changes in the percentage of the various sized lymphocytes in thoracic lymph (table 3), after several days of fistula drainage, is not clear. Wiseman<sup>14</sup> states that size is not strictly a function of age in lymphocytes. He regards the degree of basophilic staining of the cytoplasm as the more evident and reliable indication of the youth of the cells. In our experiments there appeared to be after several days of fistula drainage an increase in the percentage of cells showing a deeply basophilic cytoplasm. From the observations made by Wiseman, these findings might be interpreted as indicating that the drop in lymphocyte output through the fistula was at the expense of the older, less basophilic, cells.

#### SUMMARY

Thoracic duct fistulae in dogs created under local anesthesia and draining continuously from two to eight days have been utilized for the study of lymphocyte output. Too few observations have been made for them to be statistically significant.

\* The authors would like to express their appreciation to Dr. Joseph Feldman and Dr. Henry Bunting of the Department of Pathology, Yale University School of Medicine, for the histologic examination of the lymph node sections.

cant. It is realized that after several days of fistula drainage, factors other than the simple loss of lymphocytes through the thoracic duct fistula may have influenced the lymphocyte output. The necessity for taking into consideration the many factors which may influence lymphocyte output in the evaluation of the effect of any single factor is emphasized.

#### ACKNOWLEDGMENT

The authors would like to express their appreciation to Dr. John H. Gibbon, Jr. and to Dr. Cecil K. Drinker for their helpful suggestions in the accomplishment of this work.

#### REFERENCES

- <sup>1</sup> CAIN, J. C., GRIBBLAY, J. H., BOLESLA, J. L., FLORE, F. U. AND MANN, F. C. Lymph from liver duct. An experimental study. *Surg. Gynec. & Obst.* **71**: 559, 1947.
- <sup>2</sup> GLENN, W. W. L., CRESSON, S. L., BAUER, F. A., GOLDSTEIN, F., HOFFMAN, O. AND HEALEY, J. E. Experimental thoracic duct fistula. Observations on the technique, the absorption of fat and fluid from the intestines and protein depletion. *Surg. Gynec. & Obst.* **86**: 200-208, 1949.
- <sup>3</sup> YOTTEY, J. M. Variation in lymphocyte production. *J. Anat.* **8**: 507, 1935-1936.
- <sup>4</sup> SANDERS, A. G., FLOREY, H. W. AND BARNES, J. M. The output of lymphocytes from the thoracic duct in cats and rabbits. *Brit. J. Exp. Path.* **21**: 254, 1940.
- <sup>5</sup> ADAMS, W. S., SAUNDERS, R. H. AND LAWRENCE, J. S. Output of lymphocytes in cats including studies on thoracic duct lymph and peripheral blood. *Am. J. Physiol.* **144**: 297, 1945.
- <sup>6</sup> YOTTEY, J. M. AND DRINKER, C. K. The cell content of peripheral lymph and its bearing on the problem of the circulation of the lymphocyte. *Anat. Rec.* **74**: 417, 1939.
- <sup>7</sup> EHRICH, W. E. The role of the lymphocyte in the circulation of the lymph. *Ann. New York Acad. Sci.* **46**: 823, 1946.
- <sup>8</sup> ROUT, F. P. An inquiry into some of the mechanical factors in the production of lymphocytosis. *J. Exp. Med.* **10**: 238, 1908.
- <sup>9</sup> DRINKER, C. K. AND YOTTEY, J. M. *Lymphatics: Lymph and Lymphoid Tissue*, p. 222, Harvard University Press, 1941.
- <sup>10</sup> ——— Personal communication.
- <sup>11</sup> BARNETT, H. M., BEST, C. H. AND RIDOUT, J. H. A study of the source of liver fat using deuterium as an indicator. *J. Physiol.* **93**: 367, 1938.
- <sup>12</sup> JACKSON, C. M. *The Effects of Inanition and Malnutrition upon Growth and Structure*. Philadelphia: P. Blakiston's Son & Co., 1925.
- <sup>13</sup> JOLLY, J. Modifications des gaoghlions lymphatiques a la suite du jeune. *Compt. rend. Soc. de Biol.* **76**: 146, 1914.
- <sup>14</sup> WHELAN, B. K. Criteria of the age of lymphocytes in the peripheral blood. *J. Exp. Med.* **54**: 271, 1931.

# THE EFFECTS OF ROENTGEN RAYS ON THE INFLAMMATORY CELLS OF THE MOUSE AND RABBIT

By WILLIAM A. TOWNSEND, M D, AND BERRY CAMPBELL, PH D

ROENTGEN RAYS have been used regularly by radiologists<sup>1-12</sup> for the treatment of a large variety of inflammatory conditions, but experimental morphologic studies, as shown below, have revealed no reliable evidence to explain why roentgen rays should be of value. We undertook this problem to determine whether morphologic alterations of the inflammatory cycle could be induced by a variety of roentgen ray doses applied at times before and after inciting an inflammatory reaction in mice and rabbits. Our quantities and times of irradiation include and extend beyond those used by most earlier workers.

Sturges and Levin<sup>14</sup> were the first to study the effects of roentgen rays on leukocytes they defined as inflammatory. They found that either intravenous yeast emulsion injections or roentgen rays, when applied separately, caused a depression of the lymphocytes in the circulating blood of frogs, whereas roentgen rays superimposed on the effect of yeast emulsion had no additional action. They concluded that if the leukocytes remaining after administration of yeast emulsion were inflammatory cells, then inflammatory cells were radioresistant.

The radioresistance of inflammatory cells was further supported by Maximow<sup>15</sup> who heavily irradiated inflammatory sites locally in the subcutaneous tissue of rabbits. The earliest examination was made eleven days following irradiation when the inflammatory site contained only leukocytes and polyblasts in a gelatinous fibrin substance. The fibrocytes had been destroyed as well as collagenous fibers, so it was concluded that either leukocytes and macrophages were extremely radioresistant or that the initial infiltration had been destroyed and replaced by a second inflammatory infiltration.

Soto, Brunschwig, and Shultz<sup>16</sup> produced subcutaneous abscesses in rabbits with a variety of bacteria and with croton oil. The animals were given whole body irradiation at intervals of 24 hours before injection and immediately, 5 hours, 24 hours, and 7 days following injection of the irritant. The dose applied was 600 r (200 KV, 25 ma, 50 cm focal distance, 1 mm Cu plus 1 mm Al filter). The abscesses were mainly observed grossly but some were observed histologically. In neither case was any specific evidence found which indicated roentgen rays were of value clinically or the cause of histologic changes.

Hayer<sup>17</sup> locally irradiated (600 r) an area on a dog's thigh immediately following the subcutaneous injection of turpentine and observed that the abscess developed as in the normal animal. A similar observation was made following local irradiation of the spleen, but whole body irradiation markedly inhibited abscess development until a later period. The reason for these results was thought to be the leukopenia following whole body irradiation.

A number of authors have observed that roentgen rays decreased the number of

From the Department of Anatomy, University of Minnesota, Minneapolis, Minn.

inflammatory cells. Mitternau<sup>11</sup> irradiated acute aseptic inflammation induced by catgut sutures in the skin and subcutaneous tissue of guinea pigs and reported a decreased number of inflammatory cells within six and twenty four hours following irradiation. This decrease was also found by Fukase<sup>12</sup> within twenty four hours following irradiation of aseptic uterine incisions in the abdominal skin of rabbits. Fukase's result was confirmed by Tannenberg and Bayer<sup>13</sup> who employed a similar technic. Mischtschenko<sup>14</sup> and his co-workers examined inflammatory exudate smears two, three, and four days following irradiation of staphylococcic inflammatory sites in rabbits. They concluded that an increased leukocytic destruction as well as an increased phagocytosis was caused by irradiation.

An increased leukocytic infiltration was found by Buhtz<sup>15</sup> within a few hours following irradiation of rabbits prepared as Fukase had done previously. Buhtz had his conclusion confirmed by Tannenberg and Bayer.<sup>16</sup> Poumeau Delille<sup>17</sup> found an increased neutrophilic infiltration into rabbits' subcutaneous tissues within two days following irradiation of multiple aseptic abscesses with smaller doses of roentgen rays than used by the preceding authors. Doses over 150 r were said by the latter author to result in decreased infiltration. Burnet de Rochebrune<sup>18</sup> reported that roentgen rays imposed upon formic acid on a rabbit's skin resulted in an exudation whereas neither roentgen rays nor formic acid had this effect when applied separately.

As a standard preparation we have used subcutaneous experimental inflammation in the mouse produced by an injected inflammatory agent. A clear description of the time sequences and cytogenic development of the acutely inflamed areolar tissue of the rabbit has been contributed by Kolouch.<sup>19</sup> For a review of the earlier literature on the inflammatory article, reference is made to the elaborate discussion in the aforementioned paper, and to the textbook of Maximow and Bloom.<sup>20</sup> Kolouch studied the inflammatory cycle of rabbits and introduced the Romanowsky-stained loose connective tissue spread which was employed by the authors of this paper. This technic, which will be described below, allowed the direct comparison of cells in the connective tissue with those of the blood smears and splenic imprints.

The nomenclature of the various inflammatory cells has varied in the past with different authors. We shall attempt to follow Maximow's nomenclature as closely as possible, but convenience suggests certain modifications. The terms ameboid and resting wandering cell have been used for the resident tissue cells which are known variously as clasmatoocytes, histiocytes, tissue macrophages, etc. To indicate the cytomorphic line from the lymphocyte to macrophage we have introduced at the suggestion of Bloom, the new term intermediate polyblast.

#### MATERIALS AND METHODS

An aseptic irritant composed of egg albumin into which a small amount of india ink as a marker had been mixed was used to incite the inflammatory cycle in the mice and rabbits. This irritant was injected in .07 to .09 cc. quantities into the loose subcutaneous connective tissue on the lateral part of the abdomen. A single injection was made into each of the mice which were then killed at appropriate intervals with ether. About 200 mice were used in experiments to standardize the inflammatory cycle preparation (see below). The rabbits were similarly treated except that severe inflammatory sites were made on each animal.

According to the technique introduced by Kolouch the loose subcutaneous connective tissue of the inflammatory site was removed placed on a clean glass slide and spread, rapidly air dried and stained with Wright Giemsa

The mice were given whole body irradiation in groups of three or more in a small cardboard box. The animals were irradiated with a 7 ma superficial therapy machine set at 100KV emitting 100 skin roentgens in 1 3 minutes with no filter and a 30 cm focal distance. The doses varied from 25 r to 1600 r to the whole body which was applied at various times preceding and following the injection of the irritant. A total of 150 mice were irradiated in these experiments

The rabbits to be locally irradiated were injected with a similar irritant in two sites one on either side of the abdomen. The tissues were removed and prepared by the method of Kolouch after the rabbits had been killed at appropriate times by injected air. Twenty four hours following injection of the irritant the rabbits were irradiated on one side only under intravenous Nembutal anesthesia. Localization was accomplished by means of a cone three centimeters in diameter having a 15 5 cm focal distance. The roentgen rays were produced by a 7 ma superficial therapy machine set at 100 kV which emitted 170 air roentgens per minute with 1 2 mm of aluminum filter

Five rabbits were given whole body irradiation with the same machine similarly filtered but emitting roentgen rays at the rate of 64 8 r per minute with the focal distance raised to 33 cm. Four sites were injected on each rabbit's abdomen for the purpose of studying morphologic variations between the various inflammatory sites

Eight rabbits were given whole body irradiation with a 15 ma deep therapy machine set at 220 kV which emitted 106 r per minute with 2 mm of copper plus 2 mm of tin filter at a 33 cm focal distance. The half value layer was 1 35 mm of copper. Four sites were also injected in each animal of this series

### THE INFLAMMATORY CYCLE OF THE MOUSE

As previous literature has not described the normal cycle of inflammation in the mouse as revealed by this method, we present the following observations as a necessary basis for the irradiation experiments. The fibrocytes (fig 1) of this species differ from those described by Maximow in the human connective tissue in having a larger number of distinct chromatin clumps in an otherwise fine reticular chromatin pattern. The fibrocyte nuclei are the largest and palest staining in the connective tissue and are round or oval in outline. The indistinctly delimited cytoplasm is slightly basophilic and more homogeneous than that of the wandering cells. The fibrocyte cytoplasm occasionally contains india ink but no other particles

The wandering cells (fig 1) differ from the fibrocytes in having smaller, more deeply staining nuclei with a heavier, denser chromatin network, the cytoplasm being more basophilic, granular, and vacuolated. The cytoplasm has a distinct boundary in the ameboid wandering cells and is indistinct in the fixed wandering cells. Some of the wandering cells begin phagocytosis in the inflammatory site within one hour. These resident phagocytes give rise to large histogenous macrophages by gradual transitional forms which show increasing cytoplasmic and nuclear volume. As the nuclear volume increases, the chromatin blends into the parachromatin until frequently the nucleus appears homogeneous. This cell is relatively more frequent in the earlier hours and is considerably less frequent after the first day of the inflammatory cycle. Kolouch called this cell the activated clasmocyte

The lymphocytes of the mouse are morphologically similar to those in the human. The nuclei are either round or indented, with chromatin clumps which blend into the parachromatin. The scanty cytoplasm is deeply basophilic and occasionally

contains small vacuoles and a few fibrils. Lymphocytes and intermediate polyblasts are very occasionally found in the normal connective tissue.

The lymphocytes first migrate to the inflammatory site about the capillaries within four hours after the application of the irritant and large numbers are present within twelve hours.

A continuous variable infiltration, as contrary to the heavy initial immigration, was seen throughout the 108 hour period of observation. Within two hours after the initial infiltration began some lymphocytes were acquiring an increased nuclear and cytoplasmic volume which characterized early intermediate polyblast development (fig. 4). In the inflammatory site the evolution of the nuclei of the lymphocyte to that of the intermediate polyblast and finally to the macrophage could be followed in detail. The clumped chromatin of the lymphocyte forms strands and gradually differentiates into a heavy reticular pattern containing several distinct chromatin blocks (figs. 5b, c). Associated with the formation of the polyblast chromatin pattern was a tendency for many intermediate polyblasts to acquire a kidney shaped nucleus (figs. 4 and 5). As the chromatin became more like that of the reticulum cell, the nucleus filled out to the round nucleus of the macrophage (fig. 5c). Both cytoplasm and nucleus increased in volume during this process with the former becoming less basophilic and more granular and spongiiform. This development of the macrophage was completed in some cells within twenty-four hours and was assumed to be from those lymphocytes first infiltrating. Due to the continual infiltration of lymphocytes (fig. 6) a complete transitional series could be seen up to 108 hours. The intermediate polyblasts even in the early stages of development were seen to be capable of phagocytosis.

Although the great majority of lymphocytes formed intermediate polyblasts, an occasional lymphocyte underwent nuclear and cellular pyknosis and fragmentation. The degeneration was identical with the degeneration of lymphocytes in the spleen as discussed elsewhere. Rarely a pyknotic or fragmented nucleus could be found in cells resembling the intermediate polyblasts or macrophages. More rarely these degenerate cells contained phagocytosed material.

The fibrocytes were seen in mitosis in both the control and inflamed tissues, and especially in the later stages of inflammation. Rarely macrophages or intermediate polyblasts were found in mitosis.

The neutrophils of the mouse differed from those found in the human in having one or several lobes which formed a closed ring. A study of neutrophil development in the mouse clearly indicated that the single lobed, thick ringed nuclei were younger than the multilobed nuclei. The single lobed cell was especially prominent in the primary infiltration of neutrophils which had begun within one hour following the injection of the irritant. Large numbers of neutrophils underwent cellular fragmentation (fig. 2) and were ingested by histogenous macrophages within a few hours following infiltration. As in the case of the lymphocytes, secondary infiltration occurred throughout the period of observation. The neutrophils were found in the normal tissue in about the same frequency as the lymphocytes.

The connective tissue of the mouse also contains mast (fig. 1c) cells which are filled with metachromatic granules which obscure the nucleus. The entire cell is



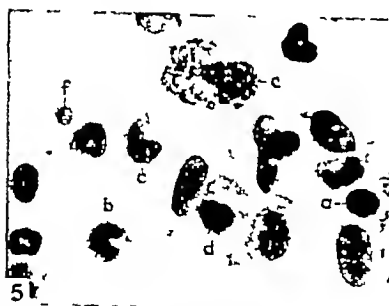
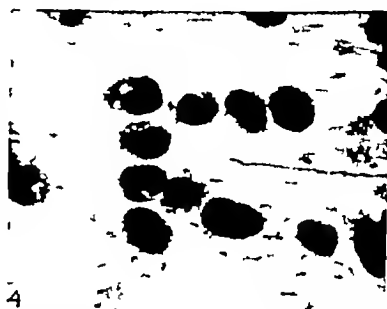
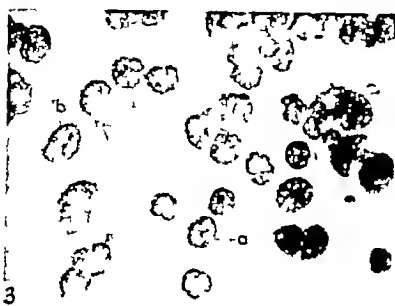


FIG. 1—Connective tissue spread from the mouse normal tissue showing fibrocytes (a) and wandering cells (b) and a mast cell (c) Wright-Giemsa.  $\times 600$

FIG. 2—Connective tissue spread from the mouse inflamed tissue showing neutrophilic infiltration and degeneration 4 hours after injection of the irritant (a) neutrophil with a thick ring nucleus (b) polymorphonuclear cell (c) pyknotic neutrophil Wright-Giemsa  $\times 600$

FIG. 3—Connective tissue spread from the mouse inflamed tissue showing lymphocytic infiltration (a) and early intermediate polyblast development (b) six hours following injection of the irritant. Wright-Giemsa  $\times 600$

FIG. 4—Connective tissue spread from the mouse inflamed tissue showing typical intermediate polyblast development 24 hours after injection of the irritant Wright-Giemsa  $\times 600$

FIG. 5—Connective tissue spread from the mouse inflamed tissue showing intermediate polyblast development and secondary neutrophilic infiltration four hours after injection of the irritant (a) lymphocyte (b) early intermediate polyblast, (c) late intermediate polyblast (d) inactive macrophage (e) degenerate lymphocyte Wright-Giemsa  $\times 600$

FIG. 6—Connective tissue spread from the mouse inflamed tissue showing a secondary infiltration of lymphocytes and neutrophils 84 hours following injection of the irritant. The small dark granules are from a ruptured mast cell Wright-Giemsa  $\times 600$

somewhat larger than the nucleus at 48 hours. These cells underwent no specific morphologic change during the inflammatory cycle although several were ruptured in the inflammatory site with consequent scattering of the granules.

#### EFFECTS OF IRRADIATION ON INFLAMMATORY CYCLE OF MOUSE

*1. Effect of irradiation on the inflammatory site of the mouse.* As the control series showed that the time of maximal lymphocytic infiltration in the inflamed areolar tissue occurred at twelve hours, this preparation was studied most thoroughly. The inflammatory cell population at this stage, in addition to the large number of lymphocytes, consists of invasive neutrophils, of lymphocytes which have differentiated into intermediate polyblasts and of histogenous macrophages. Thus the effects upon each of these cell varieties may be determined.

Irradiation of the twelve hour inflammatory site produced vacuolation in the intermediate polyblasts at dosages of 50 r to 500 r. Throughout the cytoplasm of the affected cells appeared large numbers of small clear vacuoles. A similar type of vacuole is very occasionally seen in the normal cell but never in any large numbers. Figure 7 shows the appearance of the intermediate polyblast twenty-four hours after a dose of 400 r. The vacuoles at these dosages were observed to appear at twelve hours, reach their greatest frequency at twenty-four hours, and decrease in the later stages. It would seem then that they represent a reversible alteration and hence they have been differentiated from the frank degenerative changes—fragmentation, karyorrhexis, chromatolysis, and irregularity of nuclear outline—that appear, in addition to vacuolation, at doses of 700 r or more. The preparations receiving 1200 r and 1600 r showed extensive degeneration of the intermediate polyblasts at twenty-four hours (fig. 8). This resulted in a decrease in the number of inflammatory cells relative to the fibroblasts in the tissue spreads.

The macrophages showed a greater resistance to the irradiation than did the intermediate polyblasts. Vacuolation similar to that described above, appeared at 900 r with degeneration showing at 1200 r and more (fig. 9) though differentiation in this stage cannot be made between the hematogenous and the histogenous macrophages. Consequently the relative radiosensitivities of these two cell lines cannot be ascertained. Evidence presented below indicated that up to 1200 r, the wandering cells of the tissue are unaffected.

A decrease in the number of lymphocytes and neutrophils became apparent in those preparations which had received 900 r and larger doses.

Loss of ability of the inflammatory cells to localize the irritant was evident in the preparations exposed to doses higher than 700 r. The India ink used as a marker for the egg white, was spread out over a larger area following these heavy exposures and contrary to the normal picture, tended to be phagocytosed by the fibroblasts. Whether these usually inactive cells were activated by the large doses or were responding to lack of competition of the normal scavenger cells is problematic.

*2. Irradiation of the four hour inflammatory site.* In this series only dosages of 700 r or less were tested. The effects were of the same nature as those described in the previous section though of lesser severity.

*3. Irradiation of the inflammatory site at the time of injection or at or shortly preceding*

*the time of injection* Low dosages in this series produced no alteration of the inflammatory site. At 1200 r doses, no degeneration of macrophages of tissue origin or of intermediate polyblasts was seen (fig. 10). A normal infiltration of both lymphocytes and neutrophils was found up to twenty-four hours with a decrease noticeable

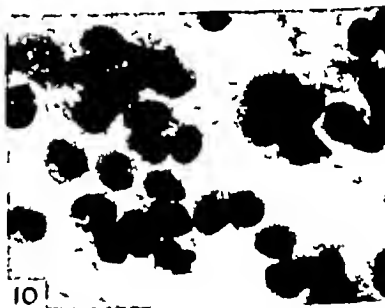
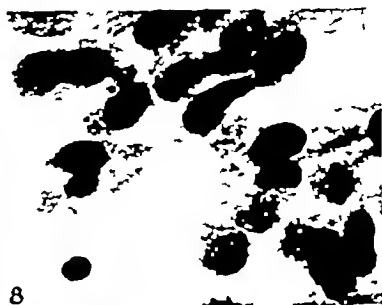
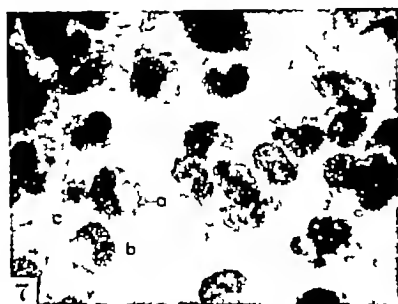


FIG. 7—Connective tissue spread from a mouse inflamed tissue showing vacuolation twenty four hours following irradiation with 400 r (a) vacuolated intermediate polyblast (b) nonvacuolated intermediate polyblast (c) degenerate lymphocyte of type seen in nonirradiated inflammation. Wright Giemsa  $\times 600$ .

FIG. 8—Connective tissue spread from a mouse inflamed tissue showing marked vacuolation and marked degeneration twenty four hours following irradiation with 1200 r. Wright Giemsa  $\times 600$ .

FIG. 9—Connective tissue spread from a mouse inflamed tissue showing indurated tissue showing marked vacuolation and marked degeneration twenty four hours following irradiation with 1200 r. Wright-Giemsa  $\times 600$ .

FIG. 10—Connective tissue spread from a mouse inflamed irradiated tissue shows normal development of intermediate polyblasts twenty four hours following irradiation with 1200 r immediately after injection of the irritant  $\times 600$ .

in the later stages. As a severe leukopenia was noted in the circulating blood in less than twenty-four hours, normal infiltration at this stage seemed the more remarkable. It was further noticed that the cells derived from the invading lymphocytes did not undergo degeneration as in the other experiments where the inflammatory site was irradiated after they had invaded the tissues. A plausible explanation for the greater resistance, under these circumstances, of the infiltrating lymphocytes and their derivatives, the intermediate polyblasts and hematogenous macrophages,

is that the mononuclear leukocytes had already been destroyed in the blood and only the mononuclear leukocytes remained.

No degeneration of the histiocytes or macrophages was seen with these massive doses, indicating the high resistance of this cell type.

2. *Irradiation of the rabbit*—*Local irradiation* Three mice only were used in this part of the experiment, one for each of the periods. They were killed twenty-four hours after the irritant was injected. All showed a greatly decreased lymphocytic infiltration and scarcity of intermediate polyblasts. The histogenous macrophages were present in normal numbers and consequently outnumbered the hematogenous inflammatory cells, a numerical relationship which is the reverse of that seen in the controls. A slight infiltration of lymphocytes was present.

TABLE 1. Summary of Rabbit Experiments

Dose (r)	Hours following injection			Average Infection	Hours following Injection		
	2	4	12		2	4	12
1600							10
1200			5	3	3		10
900							10
700	1	1	1			4	10
500							10
400						4	10
250			5	4		5	10
150					1		10
75			5	4		5	10
25							10

### EFFECTS OF IRRADIATION ON INFLAMMATORY CYCLE OF THE RABBIT

1. *Local irradiation on the inflammatory cells in rabbits* Twenty-one rabbits were irradiated locally on the twenty-fourth hour following the injection with dosages ranging from 80 r to 1000 r. Examination was made at intervals from 2 to 96 hours following the irradiation and no deviation from the controls were found with regard either to the morphology or the numerical frequency of the inflammatory cells. In addition one rabbit was exposed to 1000 r twenty-four hours before the injection and examined twenty-four hours later. No deviation from the control was noted.

2. *The effects of general body irradiation on the rabbit* Twelve rabbits were given whole body irradiation at 700 r or 1000 r and examined at intervals ranging from 5 hours to 96 hours. Of 5 animals given 1000 r (at 220 KV), 4 showed a markedly reduced infiltration of leukocytes. In the fifth only a moderate decrease in the leukocytic infiltration was seen. In all of the experiments, no abnormalities of the inflammatory, or tissue cells were observed.

### DISCUSSION

Our experiments did not reveal any acceleration of the inflammatory cycle at any dosage, though they were specifically designed for detecting such a phenomenon if

it should exist. No effects of any kind were observed at dosages below 250 r in the mouse or 1000 r in the rabbit. General body irradiation prior to the setting up of the inflammatory cycle decreased the infiltration of neutrophils and lymphocytes in dosages of 700 r or more in mice, and 1000 r in rabbits. Our evidence would indicate that the decreased infiltration is for the most part a reflection of the profound leukopenia induced. Local irradiation in the rabbits produced no such effect.

A striking resistance of all the inflammatory cells was noticed. The lymphocytes in the tissues showed no degenerative changes, indicating a great radioresistance in contrast to those observed in the hematopoietic organs but in agreement with experiments on blood.<sup>8</sup> The intermediate polyblast, which proved to be the most easily affected of the inflammatory cell population, showed no structural abnormalities, under the conditions of the experiment, in response to doses of less than 250 r and exhibited frankly degenerative phenomena at irradiations of 700 r or more. It was apparent that the macrophages of tissue origin (as well as their precursors, the wandering cells) are more radioresistant than at least some of the macrophages developing from lymphocytes. Large doses (1200 r) given at a time preceding the inflammatory stimulus resulted in no degenerative phenomena in these cells following the onset of the inflammatory cycle. Degeneration did occur in macrophages when irradiation at this dosage was performed twenty-four hours following the injection of the inflammatory agent. With 900 r, vacuolation could be observed in macrophages in those sites irradiated after the formation of the lymphocyte derivatives. No degenerative phenomena were observed in the infiltrating lymphocytes at the greatest dosage (1200 r). Neutrophils which had invaded the inflammatory site likewise proved resistant to all doses studied.

In our results, there is nothing to correlate with the conclusions of many clinicians that roentgen therapy is of great value in a number of inflammatory conditions. It is possible that a study similar to this on septic inflammation, or even the inclusion of the late stages would have made our results more confluent with previous literature. We wonder, though, whether the phenomenon of decreased infiltration which we observed in response to massive whole body exposure has been misinterpreted by some who were looking for an acceleration of the inflammatory cycle by x-rays.

Our study of the inflammatory cycle in the mouse substantiates the study by Kolouch in the rabbit. The neutrophils began invasion of the inflammatory site within one hour following injection of the irritant and reached a maximum within four to six hours. They quickly underwent degeneration and were ingested by the macrophages. Lymphocytic infiltration occurred within four hours after the injection of the irritant and immediately or very shortly afterwards began a transformation into intermediate polyblasts. The maximum number of lymphocytes was found in the tissues within twelve hours following injection of the irritant. Kolouch's paper dealt with the sequence of cell types and not quantitatively with the populations. Our findings on the variability of the secondary infiltrations of both granulocytes and lymphocytes indicate that a large number of preparations are necessary in order to deal adequately with the latter question.

## CONCLUSIONS

1. No acceleration of cell differentiation of the inflammatory cycle is induced by treatment with roentgen rays.
2. The decreased infiltration of the inflammatory site caused by massive whole-body exposures before the inflammation was set up or early in the cycle is due to the leukopenia induced in the circulating blood.
3. Of the inflammatory cells of the mouse the intermediate polyblast (a lymphocytic derivative) is relatively the most radiosensitive with structural abnormalities noticeable following 250 r. The macrophages derived from the intermediate polyblasts show alterations following doses of 700 r or more. Wandering cells, histogenous macrophages, infiltrating lymphocytes and neutrophils show no morphologic abnormalities following doses as high as 1,000 r.
4. The ability of the tissue to prevent the spread of the inflammatory agent is decreased following dosages of 700 r or more.
5. The inflammatory cells of the rabbit are markedly more resistant than those of the mouse.

## ACKNOWLEDGMENT

The authors wish to express their indebtedness to Dr. D. Wayne Townsend for aid in irradiating many of the animals.

## REFERENCES

- <sup>1</sup> BORAK J. Theories on the effectiveness of roentgen therapy in inflammatory conditions. *Radiology* 42: 249-254, 1944.
- <sup>2</sup> FREUND F. The x-ray treatment of inflammatory diseases. *Proc. Roy. Soc. Med.* 40: 111-115, 1947.
- <sup>3</sup> FRIEDMAN A. B. Superficial inflammatory diseases: treatment by radiation therapy: review of 1018 consecutive cases. *Am. J. Surg.* 25: 107-112, 1934.
- <sup>4</sup> GRANT R. A. Small doses of roentgen rays in treatment of inflammatory conditions. *New Orleans M. & S. J.* 85: 103-107, 1932.
- <sup>5</sup> HARE, H. F. Radiant treatment of benign and inflammatory conditions. *Radiology* 47: 71-73, 1946.
- <sup>6</sup> HODGES F. M. The roentgen ray in treatment of carbuncles and other injuries. *Am. J. Roentgenol.* 11: 442-44, 1924.
- <sup>7</sup> ———. Rationale of roentgen therapy in infections. *South. M. J.* 23: 259-261, 1930.
- <sup>8</sup> ———. Roentgen therapy of certain infections. *Am. J. Roentgenol.* 35: 145-155, 1936.
- <sup>9</sup> KAPLAN I. I. Treatment of non pyogenic infections with radiation therapy. *New York State J. Med.* 44: 1004-1012, 1944.
- <sup>10</sup> KELLY J. F. Roentgen rays in prevention and treatment of infections. *Radiology* 47: 478-485, 1946.
- <sup>11</sup> MAY E. A. Roentgen therapy in acute inflammatory conditions. *Radiology* 14: 411-415, 1930.
- <sup>12</sup> OSGOOD H. A. Treatment of acute and chronic inflammatory conditions by fractional doses of x-ray. *Radiology* 32: 311-314, 1939.
- <sup>13</sup> POOL H. H. Roentgen therapy with low dosage in suppurative infections. *Radiology* 4: 55-57, 1944.
- <sup>14</sup> STUBBS M. M. AND LEVIN I. Influence of radium and x-rays on the frog leukocytes. *Proc. Soc. Exper. Biol. & Med.* 18: 295-297, 1920-21.
- <sup>15</sup> MAXIMOW A. A. Studies on changes produced by roentgen rays in inflamed connective tissue. *J. Ex. Med.* 37: 319-340, 1923.
- <sup>16</sup> SOTO J. A., BRUNSCHWIG A. and SCHULTZ F. W. Experimental study of effects of x-radiation upon acute pyogenic infection of skin and subcutaneous tissues. *Surgery* 3: 593-600, 1938.
- <sup>17</sup> HAYEK E. Ergebnisse von experimentellen Studien am peripheren weissen Blutbild nach Röntgenbestrahlung. *Strahlentherapie* 50: 193-236, 1934.

- <sup>18</sup> MITTERMAIER R Experimentelle Untersuchungen zur Entzündungsbestrahlung Deutsche Ztschr f Chir 203-04 557-574 1927
- <sup>19</sup> ENKASC, S Über die Beeinflussung der traumatischen Entzündung durch Röntgenstrahlen Virchows Arch f path Anat 273 794-805 1929
- <sup>20</sup> TANNENBERG J AND BAYER L Der Heilungsvorgang von entzündlichen Veränderungen unter dem Einfluss von Röntgenstrahlen Strahlentherapie 47 408 425, 1933
- <sup>21</sup> MISCHTSCHENKO J P FOMENKO M M, FESZENKO T F LEDANOW, S N AND MOROATSCHOW A. W Experimentelle Begründung der Röntgentherapie akuter entzündlicher Prozesse Strahlentherapie 52. 464-470 1935
- <sup>22</sup> BUHTZ H Beiträge zur pathologischen Physiologie der Entzündung über die Beeinflussung der traumatischen Entzündung durch Röntgenstrahlen Frankfurt Ztschr f Path 44 57-71 1932
- <sup>23</sup> POUMEAU DELILLE, G Action de la radinotherapie à petites doses sur l inflammation aseptique Compt rend Soc de biol 235 495-497 1941
- <sup>24</sup> BURNET DE ROCHEBRUNE W F H Experimentelle Untersuchungen über die Wirkung kleiner Röntgendosen auf normale und auf leicht entzündete Kaninchenhaut Dermatologica 47 1-11 1943
- <sup>25</sup> KOLOUCH F Lymphocyte in acute inflammation Am J Path 35 413-428 1939
- <sup>26</sup> MAXIMOW A A AND BLOOM W A Textbook of Histology ed 4 Philadelphia W B Saunders Co 1944 Pp 95-98
- <sup>27</sup> SCHREK R Radiosensitivity of lymphocytes and granulocytes in vitro according to method of unstained cell counts Proc Soc Exper Biol & Med 58 285-286 1945
- <sup>28</sup> SPURLING R G AND LAWRENCE, J S Direct effect of radium on leukocytes Am J M Sc. 169. 157-160 1925

# A PRELIMINARY STUDY OF THE RELATIONSHIP BETWEEN LACTOBACILLUS LACTIS DORNER RESPONSE AND CLINICAL ANTI-PERNICIOUS ANEMIA ACTIVITY OF LIVER EXTRACTS

By WILFRID F. WHITE, M.S., JOHN R. MOTIL, M.D., AND EDWIN E. HAYS, Ph.D.

SINCE the isolation of vitamin B<sub>12</sub> in crystalline form was announced in this country,<sup>1</sup> several investigators have published the results of clinical studies with the pure material on pernicious anemia patients. These studies, while incomplete, leave little room for doubt that vitamin B<sub>12</sub> is a potent anti-pernicious anemia factor. Allowing for individual variation of patients, the responses have been proportional to the amounts of vitamin B<sub>12</sub> used and in turn proportional to the microbiologic activity as measured by *Lactobacillus lactis* Dorner. Thus, the picture with regard to clinical response to pure vitamin B<sub>12</sub> is rapidly becoming clear. However, in view of the observed wide variation in the *Lactobacillus lactis* Dorner potency exhibited by commercial parenteral liver extracts,<sup>1</sup> we have felt the need for an investigation of the relationship between the microbiologic response obtained with this micro-organism and the clinical response to both oral and parenteral liver extracts.

We have accumulated assay data on 69 pernicious anemia cases in which a variety of liver extract preparations were used. The preparations were prepared by several different methods, but each was carefully assayed microbiologically by means of the *Lactobacillus lactis* Dorner organism. We believe that the data accumulated are sufficiently pertinent to justify publication of these preliminary results.

**Samples\*** The samples can be classified as follows: (1) Experimental oral extracts made from several crude liver fractions including the supernatant of autolyzed whole liver, whole aqueous extracts of liver and 70 per cent alcohol soluble fractions of aqueous liver extract; (2) experimental crude parenteral solutions in the 2-5 U.S.P. units per cc. classification made from the 70 per cent alcohol soluble fraction of aqueous liver extract; (3) experimental refined parenteral solutions in the 15 U.S.P. units per cc. classification and prepared by several different experimental processes. The preparations for oral administration were given in three equal daily doses. The crude parenteral solutions were given twice a week and the more highly refined concentrates were given by a single parenteral injection every fourteen days.

**Clinical Testing** All subjects tested were anemic either as a result of no previous liver therapy or were in relapse due to a lapse in treatment. The latter group of patients had not received liver or arsenic for the period recommended by the Anti-Pernicious Anemia Board of the U.S. Pharmacopoeia.<sup>2</sup> Before liver therapy was started, each patient was hospitalized until the physician in-charge was satisfied that the diagnosis of Addisonian pernicious anemia was correct. In all but one or two cases hospitalization was continued at least until the end of the second week of treatment. In many instances this was continued through the third week.

**Microbiologic Test** This work was done under the supervision of Mr. J. F. Roland in our Nutrition Section. The *Lactobacillus lactis* Dorner organism was handled essentially as described by Shorb<sup>3</sup> using a 42-hour incubation period.† Details of the method will be published in the near future. The results have

From the Chemical Research and Development Department, The Armour Laboratories, Chicago, Ill.

\*All samples referred to in this paper were prepared from pork liver.

†No enzymatic release of *Lactobacillus lactis* Dorner activity was attempted.



TABLE 1—Summary of Clinical Data

Assay No	Date Started	LLD units per 2 wks X 1000	Initial RBC	1st week		R. B. C 2nd week		3rd week		Reticulocyte Peak			Clinical Response
				Found	Expected	Found	Expected	Found	Expected	%	Expected	Day	
Parenteral Extracts													
63	1/21/48	11	1 50	1 36	2 1					4 6	18	8	Poor
64	1/17/48	21	1 58	2 08	2 35					5 6	15		Poor
67	1 16/48	11	2 26	2 0	2 55	2 18	3 25			2 0	9 6	6	Poor
69	2/2/48	56	3 0	3 93	3 35	4 01	3 65			4 0	4 0	6	Full
73	2/11/48	88	1 75	1 72	2 35	2 2	2 8	3 35	3 35	25 6	15		Moderate
75	2/3/48	60	1 23	1 23	1 9	2 06	2 5	2 23	3 1	30 6	24	8	Poor
76	3/1/48	69	2 26	3 12	2 7					11 3	11	5	Full
80	2/22/48	38	1 3	1 63	2 0	2 5	2 6	3 48	3 70	15 2	20		Moderate
81	2/7/48	33	1 5	2 2	2 15	2 4	2 65	3 2	3 25	3	18	6	Full
84	2/22/48	33	2 5	2 8	2 95	3 1	3 3	3 3	3 5	13	7	5	Moderate
96	4/27/48	65	2 3	2 6	2 5	3 75	3 25	3 75	3 75	14	8 6	8	Full
109	7/15/48	108	0 65	1 20	1 14	4 5	2 1	3 25	2 8	52	30	8	Full
110	7/15/48	182	2 15	2 59	2 6	2 93	3 1	3 54	3 5	18 4	10	7	Full
A	9/20/46	65	1 01	1 22	1 7	2 22	2 3	2 61	3 0	2	41		Moderate
B	7/20/45	65	2 5	3 05	2 95	3 18	3 33			2 6	11		Moderate
C	5/20/46	76	2 36	2 38	2 85	3 45	3 20	3 57	3 *				Full
D	3/13/46	24	2 20	2 0	2	2 9	3 0			10 9	15		Moderate
E	8/20/46	34	2 50	2 86	2 9	3 05	3 3			5 3	11		Moderate
F	6/17/46	34	1 33	1 77	1 9	1 92	2 5			10 2	31		Poor
G	4/30/46	48	1 5	2 53	2 1	2 36	2 65	2 88	3 25	29	28		Moderate
51	11/4/41	92	1 43	2 17	2 05	3 0	2 60			40	19	6	Full
53	11/7/41	40	1 54	1 60	2 15	1 96	2 70			11 6	17	8	Poor
65	1/7/48	12	2 58	2 3	3 0	2 34	3 4			9 0	6 2	8	Poor
66	2/9/48	27	2 02	2 0	2 5	1 90	3 0			13	12		Poor
68	2/16/48	23	1 38	1 8	2 05	2 93	2 6			40 2	20	7	Full
70	2/20/48	55	1 53	1 49	2 15	1 55	2 0	2 0	3 25	6 0	16	5	Poor
71	3/8/48	96	3 64	3 41	3 90	4 17	4 10			5 0	4	5	Full
74	2/3/48	55	50	6	1 3	1 1	2 0	1 8	2 75	35 8	30	1	Poor
79	3/23/48	20	1 55	2 08	2 1					25 2	18	8	Full
89	4/12/48	25	1 96	2 07	2 4	2 49	2 9	3 09	3 4	9 0	13 5	8	Moderate
94	4/21/48	60	1 29	1 73	1 95	1 87	2 55			16 8	22	6	Poor
85	3/19/48	100	2 2	2 47	2 7	3 32	3 1	3 5	3 6	8 0	9 6	5	Full
86	3/23/48	63	2 65	2 9	3 0	3 0	3 4			8 2	5 8	6	Moderate
87	4/6/48	30	1 3	1 5	1 95	1 65	2 5	1 5	3 1	15 7	22	6	Poor
90B	4/24/48	60	1 8	2 2	2 3	2 8	2 55	3 4	3 7	33 2	14 5	8	Full
91	4/14/48	30	2 6	2 8	3 0	3 34	3 35						Full
92	4/15/48	20	1 1	1 7	1 8	1 5	2 4			23	27	6	Poor
93B	4/26/48	90	1 9			2 93	2 9			14 6	13	8	Full
103	5/31/48	60	1 9	2 5	2 5	2 3	2 9	2 2	3 4	5 2	13	7	Poor
107	7/10/48	80	2 0	2 55	2 53	2 95	3 0			10 6	11 4	7	Full
108	7/12/48	100	0 8	0 97	1 55	1 49	2 7	1 93	2 9	18 5	30	6	Poor
111	8/6/48	50	0 85	1 5	1 6	1 1	2 2	1 4	2 9	31 5	30	5	Poor
112	7/18/48	75	1 0	1 30	1 7	1 16	2 3			32	41		Poor
113	8/6/48	50	2 8	3 15	3 15	3 4	3 5			4 4	0	6	Full
114	8/20/48	50	1 35	2 15	2 0	2 4	2 55			22 6	31	6	Moderate
118	9/11/48	120	2 7	2 75	3 0	3 1	3 4			8 7	9 4	6	Moderate
123	9/30/48	84	2 2	2 67	2 7	2 9	3 1	4 0	3 6	24 6	15	6	Full
126	11/18/48	170	90	1 7	1 6	2 7	2 25	3 2	2 95	32 6	41	4	Full
128	6/24/48	25	1 85	2 18	2 4	2 21	2 9			12 3	21	6	Poor
130	11/26/48	72	1 38	1 94	2 0	2 2	2 6			22 5	30	3	Moderate
131	12/11/48	68	1 95	2 65	2 5	2 53	2 95			16 4	19	3	Moderate
133	9/22/48	60	1 7	1 9	2 25					7 1	23	6	Poor
135	12/23/48	35	1 6	2 2	2 2	2 25	2 7	2 6	3 25	17 6	26	6	Poor
137	1/26/49	70	2 6	2 9	2 95	2 66	3 35	2 88	3 75	15 9	10 6	5	Poor
138	2/9/49	96	1 6	2 45	2 2	2 8	2 7			18	25	5	Full
139	1/7/49	100	1 65	2 05	2 2	2 65	2 75			10 4	24	6	Moderate
140	1/22/49	100	2 2	2 5	2 7	2 7	3 1			12 2	15	5	Moderate
141	2/5/49	70	2 0	2 1	2 5	2 5	3 0			19 0	18 5	7	Poor
142	2/15/49	96	1 35	2 6	2 15	2 6	2 7	2 8	3 3	25 6	26 5	6	Moderate

Assay No.	Date Admin.	LLD units per cc					Lactobacillus		Clinical Response
		1	2	3	4	5	6	7	
57	1/13/48	35	4	61	10	8			Poor
58	1/13/48	3	14	14	33	40	14	4	Poor
59	1/14/48	3	24	41	118	11	14	38	Poor
15	1/14/48	4	1	2	1	1	1	1	Moderate
115	1/13/48	4	11	14	14	14	14	17	Poor
121	1/12/48	4	3	1	1	1	1	10	Poor
14	1/14/48	13	14	14	14	14	14	13	Full
11	1/14/48	1	1	1	1	1	1	27	Poor
83	3/1/48	1	1	1	1	1	1	47	Moderate
2	3/1/48	1	1	1	1	1	1	8	Full

TABLE 2.—Summary of Microbiologic Assay versus Clinical Patient Response

TABLE 2.—Summary of Microbiologic Assay versus Clinical		Evaluation of Clinical Responses No. of Cases		
Clinical Assay Nos.	Microbiological Assay LLD units per 2 weeks X 1000	Low	Moderate	Full
<i>Grade Parenteral Solutions (14)</i>				
63 64 67 80 81 84	Less than 49	3	2	1
69 75 76 96 A	50-75	1	1	3
73	76-100	0	1	0
109 110	Over 100	0	0	2
<i>Refined Parenteral Solutions (45)</i>				
D E F G 68 87 91 92 53 65 66 89 128 135	Less than 49	8	4	2
B 70, 74 94 86 90B 103 107, 111 112, 113 114	50-75	9	5	3
130 131 133 137 141	76-100	1	3	6
C93B 108 123 51 71 138, 139 140 142	Over 100	0	0	4
79 85 118 126				
<i>Oral Samples (10)</i>				
97 98 99 101	Less than 3000	4	0	0
115 121	3100 to 5000	2	0	0
72, 83 105	5100 to 6000	0	2	1
124	Over 6100	0	0	1

been expressed in terms of a 15 USP units per cc parenteral liver solution which had previously been assayed clinically and found to be moderately active. This standard was arbitrarily assigned a value of 75,000 *Lactobacillus lactis* Dorner (LLD) units per cc.\*

Table 1 gives a summary of the clinical data obtained, together with the total number of *Lactobacillus lactis* Dorner units administered to the patients during a two week period. Table 2 summarizes the correlation between the number of LLD units administered over a two week period and the clinical response as judged by the general criteria suggested by the Anti-Pericious Anemia Advisory Board of

\* This liver standard contains 4.9 micrograms of vitamin B<sub>12</sub> per cc as measured by microbiologic assay.

the U S Pharmacopoeia<sup>5</sup> as being important in evaluating the anti-pernicious anemia potency of liver extract preparations

The data presented in table 1 and summarized in table 2 suggest the following conclusions

1 From the limited series of cases reported upon experimental oral liver preparations, it appears that between 5,000,000 and 6,000,000 LLD units over a fourteen day period (350,000 to 450,000 LLD units daily) in the form of liver extract appear to be required to obtain an optimal hematologic, neurologic and general clinical response in cases of pernicious anemia. This amount of LLD activity appears to be required regardless of the manner in which the liver extract preparation is treated during processing

2 Although the series of cases reported using various experimental parenteral liver extracts is not large, it appears that the intramuscular injection of approximately 100,000 LLD units over a fourteen day period (representing approximately 6,500 LLD units daily) in the form of liver extract is required to obtain an optimal hematologic, neurologic and general clinical response in cases of pernicious anemia. Again this amount of LLD unitage appears to be required regardless of the manner in which, or the degree to which, liver extract is fractionated or refined

3 Taking into account the fact that the data presented are derived from a comparison of two bioassay methods (each of which has its own inherent variables), it is reasonable to conclude that there is a correlation between *Lactobacillus lactis* Dorner assay potency and human anti-pernicious anemia assay potency in liver extract preparations

#### ACKNOWLEDGMENTS

The clinical testing of liver fractions was under the supervision of the following physicians whose cooperation is gratefully acknowledged: Harold L. Alt, Northwestern University Medical School; Quinn B. DeMarsh, University of Washington Medical School; Robert A. Green, University of Minnesota Medical School; R. A. Hughes, St. Mary's Hospital, Montreal; C. M. Huguley, Emory University Medical School; Carl Johnson, St. Luke's Hospital, Chicago; Louis Limarzi, University of Illinois Medical School; Edward S. Mills, Montreal General Hospital; T. R. Smith, University of Chicago Medical School

#### REFERENCES

- 1 RICKES E. L., BRINK N. G., KONIUSZY F. R., WOOD T. R. AND FOLKERS A. Crystalline vitamin B<sub>12</sub>. *Science* 107: 396, 1948
- 2 WEST, R. Activity of vitamin B<sub>12</sub> in Addisonian pernicious anemia. *Science* 107: 398, 1948
- 3 SPIES, T. D., STONE, R. E. AND ARAMBURU T. Observations on the anti-anemic properties of vitamin B<sub>12</sub>. *South M. J.* 41: 522, 1948
- 4 SHORR, M. S. Unidentified growth factors for *Lactobacillus lactis* in refined liver extracts. *J. Biol. Chem.* 169: 455, 1947
- 5 U. S. P. Anti-Anemia Preparations Advisory Board. Third Announcement. *J. Am. Pharmaceutical Ass.* 53-55, 1940

## PERNICIOUS ANEMIA IN CHILDHOOD

REPORT OF CASE 1. SIX YEAR OLD GIRL WHO DID NOT RESPOND TO REFINED LIVER EXTRACT,  
FOLIC ACID AND VITAMIN B<sub>12</sub> IN SUCCESSIVE RELAPSES

B. R. WELLS, DAVE, M.D., RICHARD M. CHRISTIAN, M.D.,  
DONALD M. LEVY, M.D. AND LAWRENCE I. YOUNG, M.D.

**THE PURPOSE** of this paper is to present an unusual case of megaloblastic anemia in relapse in a 6 year old child who was treated successfully with vitamin B<sub>12</sub>. This case is of particular interest because the patient had exhibited similar responses to parenteral administration of refined liver extract and folic acid during previous relapses.

Megaloblastic anemia of infancy has recently been defined by Zeulzer and Ogden.<sup>1</sup> These investigators reserved this term for that group of children under 2 years of age who showed bone marrow changes identical with pernicious anemia and recovered either spontaneously or after a single course of liver or folic acid therapy.

Only a few cases of megaloblastic anemia in children requiring continuous treatment with antipernicious anemia factor have been reported. Jonsson<sup>2</sup> listed only 7 cases in addition to 2 of his own that he felt met the requirements, and Peterson and Dunn<sup>3</sup> accepted only 3. These cases have been amply reviewed by Jonsson,<sup>2</sup> Peterson and Dunn<sup>3</sup> and by Benjamin.<sup>4</sup> An additional case representing the same disease complex has been reported by Waagstein.<sup>5</sup> Prompt response followed administration of liver extract or folic acid in these cases, but response to vitamin B<sub>12</sub> in such patients has not yet been reported.

Vitamin B<sub>12</sub>, a pure crystalline antipernicious anemia factor, isolated from liver, was announced in a series of three papers<sup>6-8</sup> published in April 1948 in this country and by Smith<sup>9</sup> in England at about the same time. Spies<sup>10-12</sup> and associates demonstrated the effectiveness of vitamin B<sub>12</sub> in cases of pernicious anemia in adults, macrocytic nutritional anemia, tropical sprue and non-tropical sprue. Berk et al.<sup>13</sup> reported that vitamin B<sub>12</sub> was effective in the treatment of the neurologic manifestations in pernicious anemia. Hall and Campbell,<sup>14, 15</sup> after treating 11 patients with pernicious anemia, state that vitamin B<sub>12</sub> was as effective as liver extract in managing the hematologic and neurologic aspects of the disease. Lohby<sup>16</sup> reported that vitamin B<sub>12</sub> alone was ineffective in the treatment of megaloblastic anemia of infancy, but when small amounts of folic acid were added a response was obtained. He concluded that vitamin B<sub>12</sub> alone could not catalyze nucleoprotein metabolism to the complete stage necessary for red blood cell formation.

Bethell and his associates<sup>17</sup> have shown that B<sub>12</sub> is present in the feces of patients

From the Departments of Pediatrics and Medicine The University of Rochester School of Medicine and Dentistry Rochester N. Y.

The observations described in this paper were made under a contract with the University of Rochester Atomic Energy Project and were supported in part by the Hochstetter fund.

with pernicious anemia in relapse in approximately the same quantities as in normal individuals. Fecal extracts from a patient with pernicious anemia in relapse when given intramuscularly to an untreated case of pernicious anemia, produced significant hematologic and clinical response. Bethell states, however, that vitamin B<sub>12</sub> is ineffective in macrocytic anemia of pregnancy and the puerperium. It appears that vitamin B<sub>12</sub> may be the principal anti pernicious anemia factor in liver, although information thus far accumulated is not conclusive.

### REPORT OF CASE

M. D. unit # 189620 was born in Rochester Municipal Hospital on 4/4/42 following 9 months gestation period and a normal labor. The birth weight was 3400 grams. The cord blood Wassermann was negative. The neonatorium was not remarkable; no cyanosis, jaundice or pallor was noted. There were 4 normal siblings ages 12, 10, 8 and 3 years. The father and mother were in good health and of Sicilian extraction.

The child did well and showed no abnormalities in growth and development until the age of 16 months. In the fall of 1943 she was admitted to the Strong Memorial Hospital on three occasions because of progressive irritability, anorexia, pallor and weight loss. On each occasion marked anemia with red blood counts as low as 1.68 million and hemoglobin as low as 4.4 grams per cent were found. She was treated symptomatically and given several small whole blood transfusions. No definite diagnosis was made.

#### *Fourth Hospital Admission on 11/20/44*

**Interval History.** The patient during the six weeks between the third and fourth admissions was moderately improved for about one week, after which there was progressive return of symptoms. Lassitude and drowsiness were noted for two weeks prior to admission and edema around the eyes was noted for several days before admission.

**Physical Examination.** She was afebrile but pale, irritable and appeared chronically ill. The liver and spleen were not palpable and the neurologic examination was not remarkable. The examination was otherwise entirely negative.

#### *Laboratory. Figure 1*

**Course.** Three days after admission therapy with refined liver extract\* was started, 1 cc (15 units) weekly for 5 injections. A maximum reticulocyte response of 35 per cent was obtained on the fifth day of treatment. She was discharged on the thirtieth hospital day when the red blood cell count was 4.4 million and hemoglobin was 12.5 Gm.

The child was followed in the Out Patient Department for four months during which time she received two injections of refined liver extract. There were no complaints and the child gained 6 pounds in weight during this interval. She failed to return and was not seen for nine months until April 1945 when her symptoms recurred and it was found that the hemoglobin had fallen to 7.8 Gm. per cent. A course of six weekly injections of refined liver extract was started and in June 1945 she had no complaints, was cheerful and eating well and the hemoglobin was 13.6 Gm. per cent. She again failed to return for observation and therapy.

#### *Fifth Hospital Admission on 4/19/45*

**Interval History.** Patient did well for about five months following the liver therapy of the previous spring. Over the five months preceding the present admission she showed a gradual return of symptoms.

**Physical Examination.** The patient appeared chronically ill, was irritable and showed a yellowish sal low color to the skin. The liver was palpable two fingerbreadths below the costal margin but the spleen was not felt. Except for a soft blowing apical systolic murmur the remainder of the physical examination was negative.

**Laboratory.** Laboratory findings not included in table 1 were as follows. Vitamin A absorption and glucose tolerance tests were normal. Duodenal drainage revealed normal juices. G-1 series showed a probable deficiency pattern. The electrophoretic pattern was normal. Sickling tests were negative.

\* Reticulogen—Eli Lilly and Company



in the bone marrow are shown in figure 1. She was discharged on the fourteenth hospital day with red blood cell count of 3.7 million and hemoglobin of 12 grams per cent.

The patient is currently receiving 15 units of refined liver extract every three weeks. Clinical and laboratory findings are normal at the time of this writing.

### DISCUSSION

The similar responses to refined liver extract, folic acid and vitamin B<sub>12</sub> are illustrated in the upper three graphs of figure 1. Maturation of erythroid elements

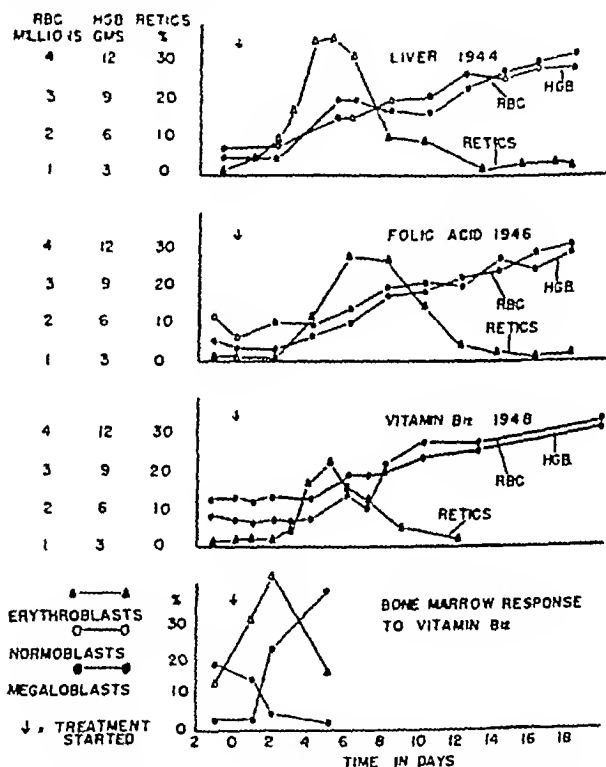


FIG. 1.—Hematologic response to therapy with liver, folic acid and vitamin B<sub>12</sub>.

in the marrow following administration of B<sub>12</sub> is also shown graphically in figure 1 and photomicrographs of the maturing erythroid cells are shown in figure 2. The need for continuous therapy to prevent relapse is apparent in view of the five relapses that have occurred during the four years of observation.

The criteria necessary for the diagnosis of pernicious anemia in childhood according to Peterson and Dunn<sup>2</sup> are (1) macrocytic anemia, (2) arrest of maturation of bone marrow at the megaloblastic level, (3) specific response, i.e., reticulocytosis after liver therapy, (4) need for continuous therapy to maintain a continuous remission, (5) histamine resistant achlorhydria.

There is general agreement concerning the first four of these criteria, but other





the fact that a small amount of free hydrochloric acid was present in the gastric juice after injection of histamine. Prompt hematologic response was obtained following administration of refined liver extract, folic acid and vitamin B<sub>12</sub> in successive relapses.

## REFERENCES

- <sup>1</sup> ZUELZER W W AND OGDEN F N Megaloblastic anemia in infancy. *Am J Dis Child* 71 211-243 1946
- <sup>2</sup> JONSSON B J Pernicious anemia in children. *Acta Paediat Suppl* 1 32 485-500 1944-45
- <sup>3</sup> PETERSON, J C, AND DUNN S C Pernicious anemia in childhood. *Am J Dis Child* 71 252-268 1946
- <sup>4</sup> BENJAMIN B Infantile form of pernicious (Addisonian) anemia. *Am J Dis Child* 75 143-189 1948
- <sup>5</sup> WAAOSTEIN P H D A case of pernicious form anemia in a child nineteen months old. *Acta Med Scand* 121 547-554 1948
- <sup>6</sup> RICKES E L, BRINO W G, KORUSZY R D, WOOD, T R, AND FOLKERS A. Crystalline vitamin B<sub>12</sub>. *Science* 107 396-397, 1948
- <sup>7</sup> SNORT M S Activity of vitamin B<sub>12</sub> for the growth of *Lactobacillus acidus*. *Science* 107 397-398 1948
- <sup>8</sup> WETT R Activity of vitamin B<sub>12</sub> in Addisonian pernicious anemia. *Science* 107 398-400 1948
- <sup>9</sup> SMITH E L Purification of antipernicious anemia factors from liver. *Nature* 161 638-639 1948
- <sup>10</sup> SPIES T D, STONE R E AND ARAMBURU T Observations on the anti anemic properties of vitamin B<sub>12</sub>. *South M J* 41 522-523 1948
- <sup>11</sup> ——— LOPEZ G G, MILANES F, TOCA R L AND CULVER, B Observations on the hemopoietic response to persons with tropical sprue to vitamin B<sub>12</sub>. *South M J* 41 423-425, 1948
- <sup>12</sup> ——— SUAREZ R M, LOPEZ G G, MILANES F, STONE R E, TOCA R L, ARAMBURU T, AND KARTUS S Vitamin B<sub>12</sub> as a therapeutic agent. *J A M A* 139 521-524 1949
- <sup>13</sup> BERK L, DENNY-BROWN D, FINLAND M AND CASTLE W B Effectiveness of vitamin B<sub>12</sub> in combined system disease. *New England J Med* 239 328-330 1948
- <sup>14</sup> HALL B E AND CAMPBELL D C Vitamin B<sub>12</sub> therapy in pernicious anemia I Effect of hemopoietic system. A preliminary report. *Proc Staff Meet Mayo Clin* 23 584-590, 1948
- <sup>15</sup> ——— AND ——— *Ibid* III Effect on the general clinical and neurological manifestations. Preliminary report. *Proc Staff Meet Mayo Clin* 23 591-594 1948
- <sup>16</sup> LUHBY A L Vitamin B<sub>12</sub> in megaloblastic anemia of infancy. To be published in proceedings of the Conference on Preservation of the Formed Elements and of the Proteins of the Blood. Harvard Medical School January 1949
- <sup>17</sup> BETHELL F H, MEYERS M C, NELSON, R B, AND SWENDSEID M E Clinical experiences with crystalline vitamin B<sub>12</sub> and with fecal extracts possessing B<sub>12</sub> activity. To be published in proceedings of the Conference on Preservation of the Formed Elements and of the Proteins of the Blood. Harvard Medical School January 1949
- <sup>18</sup> WINTROBE M M Clinical Hematology ed 2. Philadelphia: Lea & Febiger 1946 P 368

# TUBERCULOUS SPLENOMEGALY WITH THE HYPERSPLENISM SYNDROME

## A CASE REPORT

Bj H C MEREDITH, JR, M D, J Q EARLY, M D, AND WALTER BECKER, M D

ALTHOUGH secondary involvement of the spleen in tuberculosis is quite common, the clinical picture of an enlarged tuberculous spleen with little or no tuberculosis elsewhere is rare.<sup>1-10</sup> This syndrome is often referred to as primary tuberculosis of the spleen, and was first recognized by Coley in 1846.<sup>1a</sup> The term primary tuberculosis of the spleen implied that the principal location of the disease was in the spleen which acted as a focus for the dissemination of the tubercle bacilli and was responsible for the hematologic effects. Englebreth-Holm in 1938 felt that that term was misleading, as tuberculosis of the spleen must always be secondary, and insisted it be called tuberculous splenomegaly,<sup>5</sup> a term now agreed to by most writers.

Recently there has been such a case in the University of Virginia Hospital following streptomycin-treated military tuberculosis. Although there was apparent recovery from military tuberculosis, a very marked splenomegaly developed in association with leukopenia and anemia. Following splenectomy, definite clinical improvement occurred and the blood picture returned to normal. However, six weeks postoperatively, the patient developed a fulminating tuberculous meningitis and died.

## CASE REPORT

An 18 year old white male was admitted to the University Hospital on February 8, 1948. Two months previously he had first noticed a nonproductive cough associated with a dull pain in the left chest. Three weeks before admission the chest pain became more severe and simultaneously the cough increased and became productive of small amounts of yellowish sputum. Weakness, malaise, fever and severe night sweats developed necessitating confinement to bed. The patient was seen in the Outpatient Department five days before admission and an x-ray of the chest showed only accentuation of the pulmonary markings. A shaking chill occurred two days later and hospitalization was advised. There was a 25 pound weight loss.

On admission temperature was 100.5 F, pulse 120, respiration 24, and blood pressure 120/60. The patient was pale with flushed cheeks, undernourished, and showed evidence of recent weight loss. There was a generalized lymphadenopathy of small nontender discrete nodes. Examination of the chest revealed dullness to percussion in the left base posteriorly, slightly diminished breath sounds over the right lower chest, and a few small crepitant rales in both bases. The liver was just palpable and a firm spleen edge extended 7 cm. below the costal margin. A dorsal kyphosis and scoliosis to the left were also noted.

The admission blood counts are shown in table 1. A mild anemia and marked leukopenia were present. The differential smear showed an increased number of monocytes and a normal number of platelets. The urine and Wassermann were negative. A sternal marrow examination revealed an increase in immature granulocytes. Numerous sputum examinations and cultures revealed only the usual flora. Repeated blood

From the Departments of Internal Medicine and Surgery, University of Virginia Hospital, Charlottesville, Va.

cultures stool examinations and agglutinations for typhoid tularemia, brucellosis typhus and Rocky Mountain spotted fever were negative. No abnormalities were found in the spinal fluid.

Chest roentgenograms on admission revealed multiple milary opacities throughout the lungs and their appearance over a five day period strongly suggested milary tuberculosis. Abdominal x-ray films disclosed a mass in the left upper quadrant which was thought to be the spleen as well as evidence suggesting an enlarged liver.

*Course.* During the early period of hospitalization the temperature ranged between 100 F and 104 F and the patient's condition became more precarious daily. Penicillin was started empirically without effect.

During the second week gastric washings were found to be positive for acid fast bacilli on two occasions. A tuberculin skin test (1:1000) was negative and histologic examination of a lymph node biopsy showed only chronic lymphadenitis. Repeat chest x-rays demonstrated further increase in the lung markings and a progression of the soft generalized milary infiltration. The diagnosis of acute milary

TABLE 1—Hematologic Data during the Patient's Illness before and after Splenectomy

Date	RBC	Hb	WBC	Differential						Sed rate	Platelets	Clinical note
				B	S	L	M	E	B			
2-8-48	4.3	9.5	2000	13	35	29	21	1	1	25	Normal	First admission
2-14-48			4500									
2-20-48	3.6	9	4100	16	48	30	4		1			Streptomycin
3-20-48	3.8	9	1900							33		
4-12-48		11	3400							26		To sanatorium
7-1-48	4.4		1200									Fever recurrence
9-27-48	3.2	8	725	8	44	44	4			18	107,000	Second admis sion
10-26-48	3.8	9	1000	2	60	38					112,000	Third admission
10-28-48	4.5	12	2100									Transfused
10-29-48	4.6	13	11800		87	8	5					Postoperative
11-3-48	3.6	11.5	21600	4	85	9	2				266,000	To sanatorium
12-1-48	4.9	11	8100		72	17	9		2			34 days postop.

tuberculosis was considered established and streptomycin was begun in a dosage of 2 Gm. daily on the ninth day.

By the thirteenth hospital day the fever began to exhibit a downward trend. Five days later chest films showed further spread of the generalized milary opacities but the typical progressive picture of milary tuberculosis appeared altered and this was attributed to the use of streptomycin. Consequently the dose of this antibiotic was increased to 3 Gm. daily. He improved slowly and by the thirty-seventh day he was afebrile. The dosage of streptomycin was then decreased to 2 Gm. daily. Chest x-rays at this time demonstrated a beginning diminution of the milary process.

Throughout the rest of his hospital stay he remained afebrile and gained 14 pounds with notable symptomatic improvement. However the spleen remained enlarged and repeated blood studies showed a persistent leukopenia and anemia (table 1). Another tuberculin skin test was done and was found positive in the 1:10,000 dilution. Further chest roentgenograms taken on the sixty-second hospital day revealed no evidence of tuberculosis. On April 14, 1948, after 66 days of hospitalization he was discharged in an asymptomatic state to a tuberculosis sanatorium for further care. He had received a total of 107 grams of streptomycin.

While in the sanatorium although streptomycin was not continued he was afebrile, and gained 22 pounds in the next three months. Fever recurred in July and a downhill course ensued with the loss of 18 pounds in the next two months. Frequent examinations of the spleen showed progressive enlargement. Associated with this was an increase in the leukopenia and anemia (table 1).

When readmitted to the University Hospital on September 27, 1948 for further studies the patient

appeared chronically ill and had a temperature of 100 F. A smooth, slightly tender spleen could be palpated extending almost to the pubic symphysis and well across the midline of the abdomen. Blood studies again revealed an anemia, marked leukopenia, and granulocytopenia (table 1). The sternal bone marrow was hyperplastic. Bleeding time, clotting time, clot retraction, and tourniquet tests were normal. Liver function studies were normal. Urine and stool examinations were again negative. Roentgenograms of the skull, spine, hands, pelvis, and femora were negative. During this hospitalization of six days, his temperature ranged from normal to 102 F. A diagnosis of tuberculous splenomegaly was made on the basis of the history of miliary tuberculosis, fever, splenic enlargement, and the findings in the blood.

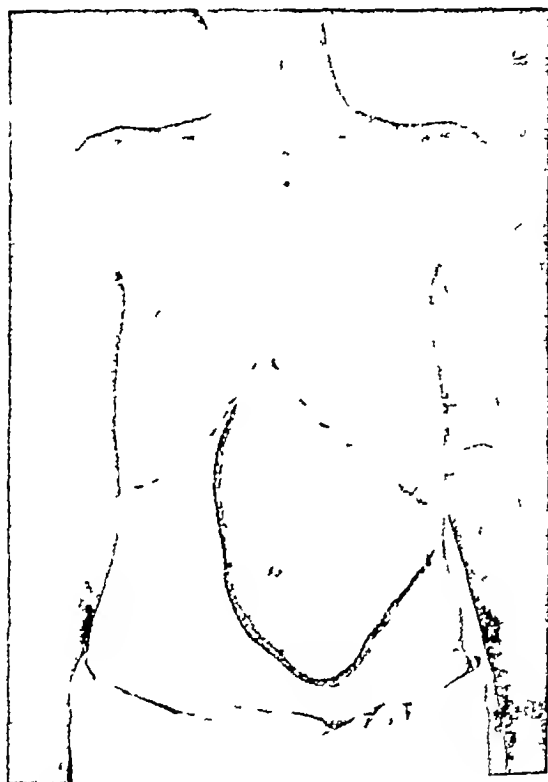


FIG. 1. OUTLINE OF THE SPLEEN PRIOR TO SPLENECTOMY

Splenectomy was advised. After receiving 1500 cc. of whole blood he was discharged to the sanatorium for further streptomycin therapy before surgery.

He remained in the sanatorium twenty-four days and during the latter twenty-one he was afebrile. However, he continued to lose weight and the splenic enlargement increased.

On October 26, 1948, he was readmitted to the University Hospital for splenectomy. Physical examination and the laboratory findings were as before. On the following day a laparotomy was performed and a very large spleen was found, filling the entire left side of the abdomen and displacing the viscera to the right. The inferior pole of the spleen extended below the sacral promontory and the capsule was extensively adherent to the surrounding structures. The liver was slightly enlarged but appeared normal. The spleen (fig. 2) was removed without difficulty and was found to weigh 3363 grams. It was smooth with a purplish grey color and had a fairly soft consistency.

On histologic examination the architecture was almost entirely replaced by tubercles with a few areas of caseation. Tubercle bacilli were demonstrated in the spleen by acid fast stains. The hilar lymph nodes were also involved with tubercles. Emulsified spleen injected into guinea pig resulted in the development of typical caseous tuberculous lesions and acid fast organisms were demonstrated in the tissues.

Within twenty four hours after the operation there was a striking increase in the white cell and platelet counts (table 1). The patient's postoperative course was uneventful except for a moderate fever. Streptomycin was continued and penicillin was added for prophylactic purposes. He was discharged to the sanatorium on the ninth hospital day for further treatment.

For the first six weeks in the sanatorium he was asymptomatic, afebrile, and gained weight. Streptomycin was discontinued at the end of four weeks. The hematologic studies remained normal and follow-up chest x-rays showed no evidence of military tuberculosis. However, during the seventh week he began

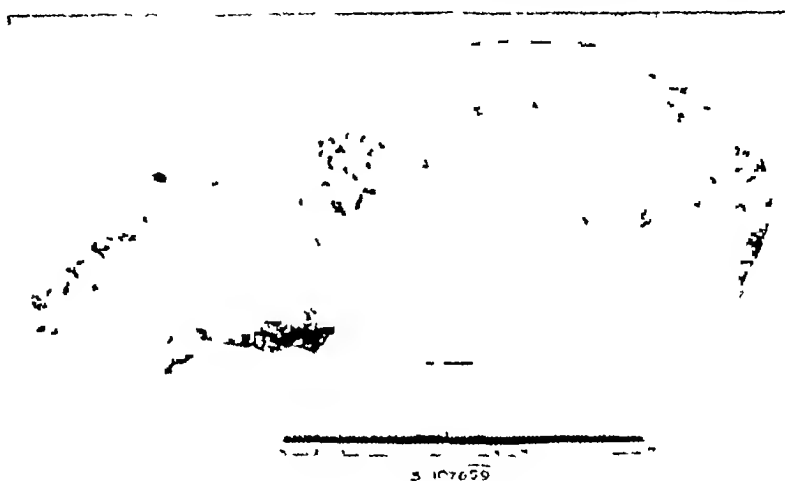


FIG. 2. CROSS SECTION OF THE SPLEEN

to run a fever and developed a stiff neck. Spinal fluid studies confirmed the clinical impression of tuberculous meningitis. Despite the resumption of streptomycin intramuscularly and intrathecally he went rapidly downhill and died within a week.

*Autopsy.* Postmortem examination revealed a healed primary tuberculous lesion at the periphery of the left lower lobe of the lung and military tuberculosis of the lungs, liver, lymph nodes and meninges. These lesions were evident grossly and microscopically and tubercle bacilli were present in smears taken from the meninges. In the cortex of the left temporal lobe there was a 5 mm area of caseation with overlying meningeal adherence. This was probably the direct source of the meningeal infection. Death was undoubtedly due to tuberculous meningitis.

### DISCUSSION

The military tuberculosis in this case at first responded dramatically to streptomycin therapy. Despite the use of this drug, the spleen, which previously had been moderately enlarged, gradually increased in size until it occupied most of the abdomen. Along with this was the development of a marked leukopenia and anemia. Three months after streptomycin was discontinued, fever returned asso-

ciated with a progressive loss of weight. A diagnosis of tuberculous splenomegaly was made and the antibiotic therapy resumed. His temperature fell to normal but he continued to lose weight and go downhill. Consequently, a splenectomy was done with the prompt reversion of the blood picture to normal and notable improvement of the patient for six weeks. However, during the seventh postoperative week he developed a fulminating tuberculous meningitis and succumbed a few days later.

Although Winternitz in 1912 felt that the blood picture in primary tuberculosis of the spleen was not constant, of the 51 cases that he collected, 42 per cent had an anemia, and 25 per cent had polycythemia.<sup>1</sup> In this series only 19 had white cell counts and 27 per cent of these had a leukopenia of 5,000 or less.<sup>1</sup> He also noted purpura in 2 cases. In the twenty years following this classic exposition, isolated reports appeared noting the association of this disease with leukopenia, anemia, thrombocytopenia, and purpura.<sup>2-4</sup> In 1931, Price and Jardine described 4 cases resembling Banti's syndrome which were diagnosed at operation or by autopsy as primary tuberculosis of the spleen.<sup>4</sup> Englebreth-Holm in 1938 after reviewing the literature and studying 4 cases of splenomegaly following military tuberculosis, observed its frequent association with anemia, leukopenia, and other evidences of bone marrow inhibition.<sup>5</sup> He concluded that tuberculous splenomegaly caused inhibition of the emission or the maturation of the blood cells in the bone marrow. In 1941, Weiner and Carter reviewed the previously reported cases of thrombocytopenia and purpura associated with tuberculous splenomegaly and added another.<sup>6</sup> The entire subject of the hemopoietic effect of this splenic disorder was reviewed briefly in 1942 by Brown, Mason, and Lucia and again by Dietz in 1946. To date less than 100 cases have been published. The occurrence in this type of splenic tuberculosis of leukopenia, anemia, and thrombocytopenia, singly or together, fits well into Dameshek's recent theory of selective and total types of hypersplenism. Dameshek postulated that the development of the one or more cytopenias in hypersplenism is due to abnormal or excessive splenic activity causing bone marrow inhibition.<sup>14, 15</sup> It is also consistent with Doan and Wright's concept of excessive destruction of blood cells by an abnormal spleen.<sup>16</sup>

The diagnosis of tuberculous splenomegaly is difficult and usually is made at the operating table or on postmortem examination. It is found most commonly in the 20 to 40 age group.<sup>2, 4, 10</sup> and the diagnosis depends on such features as a history of previous tuberculosis or exposure, splenomegaly, evidences of tuberculosis in other organs, moderate fever, evidences of bone marrow inhibition, and a downhill course. Occasionally calcium deposits can be demonstrated in the spleen by x-ray.<sup>6, 7, 12</sup> Splenic puncture with culture for the tubercle bacillus is considered of diagnostic value by some, for the organisms are quite plentiful in this organ.<sup>6, 7, 10</sup> This type of splenomegaly should be differentiated from Banti's syndrome, leukemia, lymphosarcoma, Hodgkin's disease, cirrhosis of the liver with splenomegaly, malaria, certain parasitic infections, thrombosis of the splenic vein, agnogenic myeloid metaplasia of the spleen, Felty's syndrome, and others.

Splenectomy, by unanimous agreement, is the treatment for this disorder, for

the outcome without it is generally considered to be invariably fatal<sup>1 2 4 5 10 11</sup>. The object is the elimination of the focus of dissemination of tubercle bacilli by hematogenous spread, to remove the inhibiting effect on the bone marrow, and to relieve the discomfort caused by the splenic enlargement<sup>5 10 12</sup>. In some instances the patients may not survive because of tuberculosis in other organs<sup>1</sup>. With the addition of streptomycin the prognosis should be improved. The use of streptomycin in this case did not eliminate the need for surgery.

The pathology of these spleens is quite interesting. The majority are usually huge, weighing from 1000 to 3000 grams<sup>1 5 7 9 13</sup>. The spleen in this case weighed 3,363 grams and is one of the largest reported. On macroscopic examination tubercles may or may not be seen, while caseation or abscess formation is extremely rare<sup>1 4 12 13</sup>. The histopathologic appearance is that of a very cellular pulp with numerous partly confluent miliary tubercles with little or no necrosis and only a few small malpighian bodies<sup>1 5 7 10 11</sup>. The splenomegaly is secondary to proliferation of the reticulum cells of the pulp and to the presence of tuberculous foci<sup>5</sup>.

Had the correct diagnosis been made earlier in the case reported and had splenectomy been done within the first or second month after the notable response to streptomycin, the outcome might have been different. The spleen by remaining in situ with its infection could have acted as the focus for reinfection and establishment of other foci after the organism had become resistant to streptomycin.

### SUMMARY

A case of tuberculous splenomegaly with leukopenia and anemia following miliary tuberculosis has been presented. Splenectomy was required after streptomycin failed to control the cytopenias, progressive emaciation, and splenic infection. However, following what appeared to be six weeks of marked improvement, the patient developed a fulminating tuberculous meningitis and died.

### ACKNOWLEDGMENT

The authors are indebted to Dr. Byrd S. Leavell for his suggestions in the preparation of this paper.

### REFERENCES

- 1 WINTERNITZ, M. C. Tuberculosis of the spleen. *Arch. Int. Med.* 9: 680-697, 1912.
- 2 MENTIONED BY WINTERNITZ<sup>1</sup>.
- 3 MAONAC, J. L. Surgical tuberculosis of the spleen. *Internat. Clin.* 1: 106, 1914.
- 4 KELLERT, E. Miliary tuberculosis of the spleen with thrombopenic purpura hemorrhagica. *J. A. M. A.* 96: 2193-2194, 1931.
- 5 PRICE, A. E., AND JARDINE, R. L. Primary tuberculosis of the spleen. Its clinical resemblance to Banti's disease. *Ann. Int. Med.* 4: 1574-1583, 1931.
- 6 ENGBLEBETH HOLM, J. A study of tuberculous splenomegaly and splenogenic control of the cell emission from bone marrow. *Am. J. M. Sc.* 195: 32-47, 1938.
- 7 HICKLING, R. A. Tuberculous splenomegaly, with miliary tuberculosis of the lungs. *Quart. J. Med.* 7: 236-269, 1938.
- 8 SOLOMAN, H. A., AND DORAN, W. T., JR. Primary tuberculosis of the spleen. *New York State J. Med.* 39: 1288-95, 1939.
- 9 WEINER, J. J., AND CARTER, R. F. Acute thrombocytopenic purpura hemorrhagica associated with tuberculosis (miliary) of the spleen. *Ann. Surg.* 123: 57-61, 1941.

- <sup>9</sup> BROWN J W MASON J L AND LUCIA S P Tuberculous splenomegaly—Study of a case *Ann Int Med* 17 519-28 1942.
- <sup>10</sup> COFFEY H D AND LIPTON S Primary tuberculosis of the spleen *Arch Surg* 47 478-481, 1943
- <sup>11</sup> WOODRUFF C E KELLY R G AND LEAMING M A The culturing of tubercle bacilli from the human spleen liver and kidney *Am Rev Tuberc* 48 76-93 1943
- <sup>12</sup> DIETZ C R Tuberculous splenomegaly in children *Arch Pediat* 63 168-173 1946
- <sup>13</sup> WINTROBE M M *Clinical Hematology* Philadelphia Lea & Febiger 1946 P 764
- <sup>14</sup> DAMECHER W AND ESTRELL S *The Spleen and Hypersplenism* New York Grune & Stratton 1947
- <sup>15</sup> ——— Medical progress Hematology *New England J Med* 237 434-480 1947
- <sup>16</sup> DOA C A AND WRIGHT C S Primary congenital and acquired splenic panhematopenia *Blood* 1 10-26 1946



# ABSTRACTS

JOSEPH F ROSS M D *Editor*

ABSTRACTERS

HELEN W BELDING M D Winston-Salem N C  
SHEILA T CALLENDER M D Oxford England  
GEORGE E CARTWRIGHT M D Salt Lake City  
ROGER C CRAIGS Ph D Boston  
CHARLES P EMERSON M D Boston  
SOLOMON ESTREN M D New York  
CLEMENT A FINCH M D Seattle

OLIVER P JONES Ph D Buffalo  
CONRAD MAIER M D Zurich Switzerland  
MILOŠ NETOUSEK M D Prague Czechoslovakia  
JEAN P SOULIER M D Paris  
RAMON M SUAREZ M D San Juan Puerto Rico  
WM N VALENTINE M D, Pacific Palisades Cal  
JAN WALDENSTRÖM M D Upsala Sweden

## IRON METABOLISM

THE PHYSICO-CHEMICAL REGULATION OF SIDEREMIA *S Neukomm* (University of Zurich Med School Med Polyclinic) *Acta haematol* 213 1949

Exhaustive experimental investigation leads the author to the conclusion that the fixation of iron on proteins depends mainly on the pH of the medium. The serum albumin fraction contains most of the serum iron. Hence in vivo a decrease in serum iron parallels a decrease in albumin. At the same time the erythrocyte sedimentation rate rises. There exists a mathematical correlation between the quantity of serum albumin, the sedimentation rate and the blood level of serum iron. (See also C. B. Lawell *Acta physiol Scandinav* 24 suppl 46 1947; C. E. Rath and C. A. Finch *J Clin Investigation* 23 79-85 1949; G. E. Cartwright and M. M. Winterbe *J Clin Investigation* 23 86-98 1949)

C.M.

IRON METABOLISM AND HEMOCHROMATOSIS *S Granick* From the Laboratories of The Rockefeller Institute for Medical Research New York N Y *Bull New York Acad Med* 25 403-428 1949

This review includes a comprehensive discussion of the iron compounds of the body and their properties, the mechanisms concerned in the regulation of iron absorption by the gastrointestinal mucosa, and the possible abnormalities in iron metabolism which may lead to the development of hemochromatosis.

The author's own hypothesis of the regulation of iron absorption assumes the existence of a gradient of reduction in the mucosal cell, e.g. that part of the cell nearest to the lumen of the gut is less reducing for ferric iron and that portion closest to the blood stream has a higher reducing ability. Two regulatory mechanisms, however, are probably involved. One is the determination of the amount of ferrous iron moving into the cell by a mucosal bloc which is related to the ferritin content of the mucosal cells. The other concerns the reducing ability of the cell wherein the amount of ferrous iron entering the blood stream depends on the relative redox level of the cell which in turn is a function of the amount of oxygen supplied by the blood stream.

Evidence is presented which suggests that the metabolic defect in hemochromatosis does not reside in those factors concerned with the mucosal bloc but rather is related to a greater reducing tendency of the cell for iron which permits more iron to enter the blood stream. Such a state could arise from either an increase in the effectiveness of reducing enzymes or a decrease in effectiveness of oxidizing enzymes. (See also S. Frandsen *Acta med Scandinav* 125 186-201 1947)

H W B

IRON DEFICIENCY STATES AND THEIR THERAPY WITH NEW FERROUS SALTS *B Jasinski* From the Medical Department of the Cantonal Hospital Winterthur (Switzerland) *Helv Med Acta* 26 67 1949

The author has made investigations especially with ferrous gluconate and ferrous formate. He found that small peroral doses of iron (44 mg) did not substantially alter the serum iron level of normal as well as of anemic subjects. Even larger doses of iron very rarely influenced the serum iron level of normal persons. It is the author's opinion that the hydrochloric acid in gastric secretion is of little significance for the absorption of iron preparations, yet that it plays an eminent rôle in the utilization of the iron in the food. The absorption rate of iron compounds may be assumed only in anemics. In cases

of infectious anemias and of malignant tumors the absorption of iron is inhibited i.e. we find the same rates as in normal persons. Yet there are some cases of infectious anemias that respond readily to high doses (470 mg. iron). In cases of idiopathic chronic hypochromic anemia a well tolerated iron compound is necessary. The ferrous gluconate fulfills this requirement remarkably well.

C M

## ANEMIA

THE ANEMIC STATES THEIR CAUSES AND TREATMENT. C. A. Dean and C. S. Wright. From the Division of Medical Research, Department of Medicine, Ohio State University, Columbus, Ohio. *M. Clin. North America* 33: 541-560, 1949.

The authors briefly discuss normal erythropoiesis and erythrophagocytosis, the anemias of central bone marrow origin, and finally anemias due to excessive peripheral demand (loss or destruction). Under the last subject the views of these authors on hypersplenic syndromes are discussed in some detail with illustrative examples.

G E C

THE EFFECT OF VITAMIN B<sub>12</sub> ON THE ANEMIA AND COMBINED SYSTEM DISEASE OF ADDISONIAN PERNICIOUS ANEMIA. S. R. Muttar, A. McBride and R. Tat. From the Division of Medicine of the University of California Medical School, San Francisco, Calif. *California Med.* 71: 23-27, 1949.

A study of the effect of the parenteral administration of vitamin B<sub>12</sub> on the course of 8 patients with Addisonian pernicious anemia was made. Following an initial dose of 25-50 micrograms, all patients exhibited reticulocytosis within 48 hours and peak response at about 96 hours following injection. In only 2 were secondary reticulocyte responses induced by a subsequent injection. Although maximal reticulocytoses as indicated by L. J. 22.5 formula were not obtained, the immediate conversion of megaloblastic bone marrow to a normoblastic picture and the return of erythrocytes and hemoglobin to normal levels within sixty days indicated a satisfactory remission. The authors recommend average maintenance injections of vitamin B<sub>12</sub> in the amount of 30-50 micrograms at intervals of one month. Paresthesias and other symptoms relative to early and moderately advanced combined system disease were found to disappear or be ameliorated. In a single case sensitive to refined liver, no sensitivity to vitamin B<sub>12</sub> was noted. Preliminary observations on a group of patients whose combined system disease had persisted in spite of large doses of liver extract indicated some subjective but no objective improvement in their condition following the administration of vitamin B<sub>12</sub>.

W N V

TREATMENT OF PERNICIOUS ANEMIA WITH CRYSTALLINE VITAMIN B<sub>12</sub>. R. West and E. H. Reissner, Jr. From the Department of Medicine, College of Physicians and Surgeons, Columbia University and the Fourth Medical Division, Bellevue Hospital, New York, N. Y. *Am. J. Med.* 6: 643-650, 1949.

Eleven cases of Addisonian pernicious anemia treated with parenteral vitamin B<sub>12</sub> are reported. All showed a satisfactory hematologic remission and 5 patients with combined system disease showed neurologic improvement. Different and in several cases amended dosage schedules were employed and from these several observations it was concluded that the effective parenteral dose was slightly less than one microgram daily. This approximation of a minimal effective dose is borne out by other similar studies. Any evaluation of the effectiveness of an average daily dose must, however, take into consideration the frequency and size of the individual doses.

H W B

OBSERVATIONS ON THE MACROCYTIC ANEMIA ASSOCIATED WITH PREGNANCY. T. D. Spies. From the Department of Nutrition and Metabolism, Northwestern University and the Hillman Hospital, Birmingham, Ala. *Surg. Gynec. & Obst.* 89: 76-78, 1949.

Six patients with macrocytic anemia associated with pregnancy were studied within a few weeks after delivery. The reportedly satisfactory clinical and hematologic response to folic acid is illustrated by one case report.

The author stresses the importance of realizing an association between this type of anemia in the

mother and the development of megaloblastic anemia in the infant and states that both of these can usually be prevented by treatment of the mother with folic acid during the late stages of pregnancy  
H W B

ANTI ANAEMIA ACTIVITY OF FAECAL EXTRACT FROM PERNICIOUS ANAEMIA PATIENT *S T E Callender B J Mallett G H Spray and G E Shaw* From the Nuffield Department of Clinical Medicine Oxford, and the Evans Biological Institute Runcorn Cheshire England *Lancet* 2: 57 1949

Two hundred grams of wet feces from a patient with untreated pernicious anemia were subjected to a papain digest and phenolic extraction. *L. lactis* Dorner assay of the extract showed 1 microgram per ml equivalent of B<sub>12</sub> activity. Five ml of extract given daily for five days to a second patient with untreated pernicious anemia resulted in an optimal therapeutic response. Chromatography of the extract gave a chromatogram closely resembling those shown by purified liver extracts

S C.

EXPERIMENTAL MACROCYTIC ANAEMIA IN THE RAT TREATED WITH PURIFIED LIVER EXTRACT PTEROYL-GLUTAMIC ACID AND VITAMIN B<sub>12</sub> *D G Cameron S T E Callender G M Watson and L J Wills* From the Nuffield Department of Clinical Medicine, Radcliffe Infirmary, Oxford England *Nature* 164: 188 1949

Twenty six rats made anemic by the formation of a cul de sac in the small intestine (see *Lancet* 2: 404 1948 *Blood* 4: 803 1949) were divided into four groups: a control group and groups treated parenterally with Anahaemin, pteroylglutamic acid and vitamin B<sub>12</sub> respectively. There was a significant increase in survival time with pteroylglutamic acid but not with Anahaemin or B<sub>12</sub>. Five of 6 animals treated with P.G.A. showed a hematologic remission. Two from each of the Anahaemin and B<sub>12</sub> groups of 7 and 6 animals also had a remission. The impression was that this was not fortuitous but as spontaneous remissions occurred in the control group the effect of liver extract and B<sub>12</sub> was not clear cut

S C.

NUTRITIONAL MACROCYTIC ANAEMIA IN TEMPERATE ZONES *J Rubin and C D Calnan* *Brit M J* 1: 1079-1081 1949

A case of nutritional macrocytic anemia in a mentally subnormal woman of 56 is described. The anemia failed to respond to treatment with 2 ml Anahaemin daily for ten days followed by 4 ml Flexan crude liver daily for five days but a good response was obtained with folic acid

S C.

MEGALOBlastic ANAEMIA IN COELIAC DISEASE TREATED WITH FOLIC ACID *M L Thomson H W Dalton and Vera K Wilson* From the Royal Manchester Children's Hospital *Lancet* 2: 238-240 1949

This is a detailed report of the two cases of coeliac disease associated with megaloblastic change in the bone marrow already briefly described in 1946 (Dalton et al. *Lancet* 2: 652, 1946). Treatment with folic acid resulted in general improvement with reversion to normoblastic marrow. One child remained well after discharge on 5 mg folic acid twice weekly but the other relapsed. A second relapse later led to complicating infection and death

S C.

LACTOBACILLUS LACTIS DORNER FOR THE ASSAY OF VITAMIN B<sub>12</sub> *G E Shaw* From the Evans Biological Institute Runcorn Cheshire England *Nature* 164: 186-187 1949

A technic is described for L. Dorner assay of B<sub>12</sub> in liquid medium (For details the original article should be studied). An identical shape of dose response curve is obtained with all types of liver extract, crystalline B<sub>12</sub> and Thymidine or a blend of any of these. Digesting liver with papain does not alter the shape of the curve

S C.

THE EFFECT OF ORAL THERAPY WITH COBALTOUS CHLORIDE ON THE BLOOD OF PATIENTS SUFFERING WITH CHRONIC SUPPURATIVE INFECTION *J C Robinson G W James III and R B Lark* From the Medical

Nutrition Laboratory, Department of the Army and the Department of Medicine, University of Illinois College of Medicine, Chicago, Illinois. *New England J Med* 240: 749-753, 1949

Nine patients with prolonged suppurative infections were treated for two to eleven weeks by the oral administration of 20 to 60 mg. of cobaltous chloride daily. This uniformly resulted in a reticulocytosis, an increase in the circulating erythrocyte hemoglobin and packed red cell concentration of the blood and an increase in the total red cell mass. This is a clean-cut demonstration of the stimulating action of cobalt on the bone marrow as has previously been demonstrated in animals.

The studies of Heilmeyer, Cartwright and Wintrobe and others indicate that the primary fault in the anemia of infection is a retarded rate of hematopoiesis. The clinical demonstration that cobalt acts in the opposite direction to counteract this anemia still leaves open the fundamental question: is the anemia of infection harmful to the individual or is it a useful compensatory device to conserve metabolic energy during a period of emergency? More evidence must be accumulated before it will be possible to pass opinion on the desirability of cobalt therapy for the anemia of infection.

C A F

ERYTHROPOIETIC EFFECT OF COBALT IN PATIENTS WITH OR WITHOUT ANEMIA. *L. Berk, J. H. Barbenel and W. B. Castle*. From the Thorndike Memorial Laboratory, Boston City Hospital and Harvard Medical School, Boston, Massachusetts and the Memorial Hospital for Cancer and Allied Diseases, New York. *N. Y. New England J. Med.* 240: 754-761, 1949.

Cobaltous chloride ( $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ ) was administered to 61 patients by mouth. In 7 convalescent and 8 psychotic patients, consistent reticulocytosis and elevation of hemoglobin and red count above control levels were observed. The average reticulocyte peak on a dose of 300 mg./day was 4.9 per cent and occurred between the fourth and tenth day. This dose of cobaltous chloride was not tolerated by 12 patients with pernicious anemia in remission. A good erythropoietic response was observed in 2 of 5 patients with infection. Five patients with refractory anemia and hyperplastic bone marrow gave no response. Sixteen patients with anemia due to leukemia or lymphoma were treated. There was no response in 13 of the patients. In 3 others, evidence was equivocal because of therapy of the underlying disease. One of 2 patients with hypochromic anemia due to iron deficiency and one patient with Cooley's trait responded while a patient with the anemia of liver disease was not affected. Two patients with chronic nephritis could tolerate the drug for only a few days but showed no reticulocyte response.

No serious toxic manifestations were observed but gastrointestinal symptoms of nausea and vomiting were present in 37 of the 61 patients.

It is apparent from this and other studies that cobalt provides an additional stimulus to the marrow for hematopoiesis. One might generalize from these observations that the action of cobalt is most conspicuous when the bone marrow is not under a great stimulus before treatment. As stated by the authors, the possibility that the erythropoietic action depends upon a fundamental alteration of tissue respiration indicates the need for further studies of chronic toxicity in animals.

C A F

## POLYCYTHEMIA VERA

OSLER'S CHRONIC CYANOTIC POLYCYTHEMIA WITH SPLENOMEGALY. *M. M. Wintrobe*. From the Department of Medicine, University of Utah College of Medicine, Salt Lake City, Utah. *Bull. Johns Hopkins Hosp.* 85: 75-86, 1949.

An excellent discussion of the historical aspects, clinical features, pathologic physiology and pathogenesis of polycythemia vera with particular reference to the contributions of Sir William Osler is presented. Brief mention of available therapy is made. Of particular interest is the critical evaluation of the various concepts of the pathogenesis of the condition and particularly those concepts relating to the role of anoxia. The author, while regarding the subject as an open one, is inclined to favor the view that erythremia is similar in its pathogenesis to leukemia and is not attributable to the influence of anoxia on the bone marrow.

W N V

THE CONTROL OF POLYCYTHEMIA BY MARROW IRRADIATION. A TEN YEAR STUDY ON 172 PATIENTS. *J. H.*

*Laurence* From the Division of Medical Physics and Donner Laboratory University of California Berkeley Calif J A M A 141 13-18 1949

Of 172 patients with various forms of polycythemia radioactive phosphorus was used in the treatment of some 121 in whom the diagnosis of idiopathic polycythemia (polycythemia vera) could be made. This report details the results of this form of treatment ten years after its inception.

In general the results of treatment were very satisfactory, with good relief of symptoms and signs. Of all patients treated 48 per cent required only one course of treatment of those treated during the first five years 28 per cent had only one such course and some required no further therapy for four to as long as eight years. Among other things the spleen regularly became smaller or even impalpable following treatment. Some 35 per cent of patients with polycythemia in whom the blood pressure was initially elevated showed a fall of blood pressure after  $P^{32}$  therapy. Some 70 per cent of patients with initial leukemoid blood counts showed disappearance of this feature following  $P^{32}$ .

There was no evidence of an increase of leukemia following the use of  $P^{32}$  of the 21 deaths in this series 5 (about 4 per cent) were due to leukemia. This complication was no more frequent in this group than in polycythemic patients who are untreated or treated with other methods (Fowler's solution  $\gamma$  ray, etc.).

It was of interest that thromboses seemed to be reduced following the use of  $P^{32}$  they occurred in 4.2 per cent of patients following therapy as compared with 25 per cent before treatment. Phlebotomies alone did not reduce the incidence of thromboses to this degree.

Finally the average duration of life following diagnosis was 17 years. The average age at diagnosis in this group was 50.7 years, at death 67 years. The author points out therefore that with this form of treatment the life expectancy of the newly-discovered polycythemic is as good as that of the diabetic with insulin or that of the pernicious anemia patient with liver extract. The use of radioactive phosphorus is recommended as the ideal form of treatment of polycythemia vera at the present time.

S. E.

## LEUKOCYTES

*LIVER FUNCTION DURING INFECTIOUS MONONUCLEOSIS* J. W. Brown, J. L. Sims, E. White and J. E. Clifford. From the Departments of Medicine and Preventive Medicine and Student Health University of Wisconsin Medical School Madison Wisc. Am J Med 6 321-328 1949.

Liver function tests were performed at random or in series during the course of infectious mononucleosis in 83 patients between the ages of 17 and 34. Tests included icterus index, qualitative urine urobilinogen excretion, cephalin cholesterol flocculation, thymol turbidity, prothrombin time and bromsulphalein dye retention. One or more of these tests were abnormal in 75 of the cases. The most frequently abnormal test was the cephalin cholesterol flocculation which became positive early and for a significant length of time in nearly all cases tested in series. Since it was positive more uniformly than the heterophile antibody determination it was considered a valuable diagnostic aid whenever infectious hepatitis of virus etiology could be excluded. The next most frequently abnormal test was the thymol turbidity. Abnormalities in the icterus index, urobilinogen excretion and bromsulphalein retention were also detected in a significant number of cases.

This and other studies indicate that nearly all patients with infectious mononucleosis will demonstrate abnormal liver function at some time during the course of illness.

The authors comment on the difficulty in differentiation between infectious mononucleosis and infectious hepatitis, the lack of correlation between abnormalities in liver function and duration of symptomatology in infectious mononucleosis and the need for further study of these patients particularly after recovery. Until the clinical significance of these changes in liver function is known it is suggested that patients with infectious mononucleosis be placed on the same regimen recommended for patients with infectious hepatitis.

H. W. B.

*A TRANSPLANTABLE SPLENIC TUMOR RICH IN MAST CELLS. OBSERVATIONS ON MAST CELLS IN VARIED NEOPLASMS* T. Bali and J. Farth. From the Branch 10 Laboratories of Veterans Administration Hospital Dallas, Texas. Am J Path 25 605-625 1949.

The present investigation was prompted by the finding of numerous mast cells in and about a spontaneous epithelioid-like reticulum cell splenic neoplasm in mice. No relationship could be established between neoplastic and mast cells. Miscellaneous neoplasms with the exception of some leiomyomas were in general free from mast cells. Apparently some tumors either stimulate mast cell proliferation or attract mast cells. This may be brought about by some substance present in the tumor or by a metabolite of the tumor cells. These suggest additional experimentation with subcutaneous injections of cell free extracts of certain tumors. Recent histochemical evidence links mast cell function with production of the ground substance of connective tissue and the blood clotting mechanism.

O P J

EVALUATION OF METHOD OF ENUMERATING STERNAL MARROW EOSINOPHILS *Philip Pizzolatto* From Clinical Laboratory Service Veterans Administration Hospital Department of Pathology Charity Hospital of Louisiana and Louisiana State University School of Medicine New Orleans La. *Am J M Technol* 25: 213-216 1949

The author evaluates the various methods of counting marrow eosinophils—chamber methods, direct smear, stained sections. A combination of the Levy-Newbauer chamber method with the use of May-Grunwald-propylene glycol as diluent plus the direct smear is recommended. Variations with any method even from a single procedure are appreciable.

W N V

THE LYMPHOCYTE STUDIES ON ITS RELATIONSHIP TO IMMUNOLOGIC PROCESSES IN THE CAT *C G Craddock Jr W N Valentine and J S Laurence* From the University of Rochester School of Medicine and Dentistry and the Department of Medicine of the Strong Memorial and Rochester Municipal Hospitals Rochester N Y *J Lab & Clin Med* 34: 158-177 1949

An experimental approach is presented for the study of the antibody content of lymphocytes collected from the thoracic duct lymph of cats. Using the technic described and typhoid vaccine as an antigen, no antibody could be detected within extracts of washed lymphocytes. Comparative titrations of the relative antibody content of lymph fluid free of cells and lymph containing large numbers of lymphocytes which were artificially lysed in order to release their protein content into the surrounding lymph fluid also failed to indicate the presence of any antibody within the lymphocytes. Exposure of the animals to x-ray did not significantly alter the antibody content of the cell free lymph fluid. Administration of large doses of adrenal cortical hormones failed to cause any significant alteration in the antibody content of cell free lymph fluid.

G E C

## LEUKEMIA AND MALIGNANT LYMPHOMA

TREATMENT OF CHRONIC FORMS OF MALIGNANT LYMPHOMAS AND LEUKEMIAS *L F Greer* From the Memorial Hospital for the Treatment of Cancer and Allied Diseases New York N Y *M Clin North America* 33: 527-540 1949

This is an extremely well written, easily readable general discussion on the subject of treatment of chronic forms of malignant lymphomas and leukemias. The author discusses therapy from the simple general approach of (1) the early, strictly localized disease, (2) the intermediate stage of spread of the disease, and (3) the stage of marked generalization of the disease. Individualization of treatment is stressed. Such therapeutic agents as nitrogen mustard, x-ray, urethane, arsenic, radioactive phosphorus, benzol, and folic acid antagonists are considered. The author again expresses his encouraging belief that adequate early treatment of a localized early process may offer hope for cure.

G E C.

TREATMENT OF MALIGNANT DISEASE WITH NITROGEN MUSTARD *N B Kottick, A R Pajon, M H Fisher and D K Adler* From the Second Medical Service Mount Sinai Hospital New York N Y *Ann Int Med* 30: 974-1003 1949

The authors add 64 more cases treated with nitrogen mustard to the literature. They include 13 cases of Hodgkins disease, 4 of chronic lymphatic leukemia, 2 of chronic myelogenous leukemia, 8 of lympho-

sarcoma 10 carcinomas of the lung and other miscellaneous malignancies. Many of their cases had been treated with x ray prior to mustard therapy. Data regarding the individual cases is effectively presented in tabular form. Conclusions are essentially the same as reported by others.

C.A.F.

ADVANCES IN TREATMENT OF MALIGNANT DISEASE. *C. P. Rhoads*. From the Memorial Hospital Center for Cancer and Allied Diseases, Sloan Kettering Institute, New York, N. Y. *Bull. New York Acad. Med.* 25: 271-284, 1949.

The experimental use of folic acid conjugates and folic acid antagonists in the treatment of neoplastic diseases is briefly reviewed. It is pointed out that while the status of chemotherapy of malignant disease remains essentially unchanged, a very real step has been made toward our understanding of the pathologic physiology of neoplasms. It is entirely conceivable that with further investigation of nucleic acid metabolism more effective compounds with a far more selective effect on neoplastic tissue may be found.

H.W.B.

## BLOOD COAGULATION AND HEMORRHAGIC DISEASE

THROMBOPENIA AND INCREASED CAPILLARY FRAGILITY IN HEPATIC DISEASE. *F. B. Whitsett, Jr. and A. M. Snell*. From the Division of Medicine, Mayo Clinic, Rochester, Minn. *J. A. M. A.* 140: 1071-1076, 1949.

In a study of 70 consecutive cases of various forms of liver disease, the authors noted the frequent occurrence of a hemorrhagic diathesis in those patients with parenchymatous liver disease (hepatitis, cirrhosis), whereas hemorrhagic phenomena were rare in non-parenchymatous hepatic disorders (stone stricture, carcinoma of bile ducts or pancreas). The hemorrhagic manifestations they found were not due solely or necessarily to hypoprothrombinemia, but often to thrombocytopenia and to increases in capillary fragility. They therefore studied the occurrence of thrombocytopenia and increased capillary fragility in their 70 cases: of the 29 with extrahepatic jaundice, only 4 showed these abnormalities; of the 41 patients with hepatitis or cirrhosis, 37 showed a reduction in platelets, increased capillary fragility, or both. These findings could not be correlated with hypoprothrombinemia or with jaundice.

Few data unfortunately are presented to explain these findings. What bone marrow punctures were done showed active marrows and megakaryocytes in adequate numbers; there is no note as to platelet formation from the megakaryocytes. No data are given as to other bleeding tests beyond the generalization that, usually, the bleeding time was prolonged, the coagulation time normal or slightly prolonged, and the clot retraction poor. The authors comment that the associated hypoprothrombinemia made these tests difficult to interpret, is strange. Nor can one accept the statement that the bleeding in liver disease may so often simulate that of idiopathic thrombocytopenic purpura; that every case of the latter disorder should be suspected of being thrombocytopenia secondary to liver disease (even to the point of liver biopsy) till disproven.

The data in this report, however, emphasize that the bleeding of certain patients with liver disease may be due not to a reduction of prothrombin, but to thrombocytopenia and capillary defects. The mechanism for these alterations in blood and circulatory system and the possible role of hypersplenism, are not discussed.

S.E.

HEMOPHILIA LIKE DISEASE IN WOMEN. *J. S. Hewlett and R. L. Haden*. From the Division of Internal Medicine, the Cleveland Clinic and the Frank E. Bunts Educational Institute, Cleveland, Ohio. *J. Lab. & Clin. Med.* 34: 151-157, 1949.

Two female patients with a clinical picture of hemophilia are presented. The outstanding characteristic was a prolonged coagulation time of the blood. The coagulation time of recalcified plasma was positive in both patients. When normal citrated plasma was added to blood from one of the patients, the coagulation was markedly accelerated. Tiselius protein fractionation revealed a definite abnormality in the alpha globulins in one patient and suggestive but not conclusive evidence of an abnormality in the

wound patient. In these two patients the defect was similar to that found in hemophilia but the authors are careful to call this "hemophilia like disease" and suggest that an acquired change in the plasma protein pattern might be the basis for the coagulation defect.

G E C

CHANGES IN THE PROTHROMBIN TIME INDUCED BY METHYLXANTHINES. ROLE OF THE PLASMA COFACTOR OF THROMBOPLASTIN. *F. Herrate*. From the Chemical Laboratory, School of Dentistry, University of Chile, Santiago, Chile. Arch. Biochem. 22: 345-352, 1949.

The influence of caffeine, theobromine, theophyllin and sodium benzoate on the prothrombin time of rabbits was studied, and these substances were found to shorten the prothrombin time. These results can be observed in rabbit plasma only if the latter is diluted to 5-10 per cent. If dilutions are made with human fresh plasma treated with  $Al(OH)_3$  or with fibrinogen solutions, care must be taken to make certain no thromboplastin cofactor is present in the diluent. The authors believe that these results are produced by an increase of the thromboplastin cofactor (? synonymous with Factor V [Owren] and AC-globulin [Seegers]) associated with the administration of these drugs.

W N V

THE CONTROL OF DICUMAROL THERAPY. *J. H. Olsen*. From the Department of Surgery, Presbyterian Hospital of the City of Chicago, affiliated with the University of Illinois College of Medicine, Chicago. Ill. Am. J. M. Sc. 21: 427-437, 1949.

Ninety-nine patients were treated with dicumarol. Fifty per cent of these were treated as outpatients. One-stage prothrombin methods (whole plasma, 12.5 per cent and 5 per cent plasma) were compared with the two-stage method. Wide variations between the methods were noted. With the two-stage method it was possible to maintain the prothrombin level accurately within a desired range over a period of months, with a maximum variation of 15 per cent. The one-stage method was found to be of value in estimating the summation of clotting factors in an individual blood, particularly at a time when the prothrombin as measured by the two-stage test was below 10 per cent. Bleeding occurred in 15 of the patients. In 13 of the 15 patients the prothrombin level was below 11 per cent (two-stage method) when the bleeding became apparent and stopped when the prothrombin rose to between 15 and 20 per cent. In the other 2 cases, bleeding occurred following trauma, the prothrombin being 36 per cent at the time. The results of this study suggest that a range of 10 to 30 per cent (two-stage method) is a safe, efficient level for the maintenance of plasma prothrombin.

G E C

## MORPHOLOGY

EXPERIMENTAL INFARCTION OF BONE AND MARROW. *J. H. Bragdon, L. Foster and M. C. Sosman*. From the Department of Pathology, Harvard Medical School, Boston, Mass. Am. J. Path. 25: 709-723, 1949.

Literature concerning bone and marrow infarction contains many contradictory reports based on some instances on nonphysiologic experimentation. The present series of experiments, conducted on 25 skeletally immature rabbits (2.3-3.3 Kg.), extended over a period of one day to six months. Infarcts were produced by transecting the nutrient artery and in most cases the accompanying vein of the femur. At the termination of the experiments the femurs were cleaned and roentgenograms taken. Histologic preparations were made from fragments not over 2 cm. in length after decalcification in nitric acid. Tissue changes were detected in both bone and marrow twenty-four hours following infarction and were still evident after six months. In marrow infarcts the absence of a fibrous cicatrix was striking. Fat released from necrotic cells was engulfed by phagocytic cells. Hematopoiesis was diminished or absent. An unidentified yellow material, presumably lipid, was noted in macrophages. As a rule small areas of necrotic bone were located along the inner margins of the cortex of the shaft.

O P J

HISTOCHEMICAL STUDIES IN GAUCHER'S DISEASE. *R. W. Morrison and M. H. Hack*. From the Department of Pathology, University of Illinois College of Medicine, Chicago. Ill. Am. J. Path. 25: 597-603, 1949.

For quite some time the large foamy cells derived from the reticulum and histiocytes in the spleen



liver lymph nodes and bone marrow in Gaucher's disease have been known to contain kera<sup>1</sup>so a cerebroside. This substance is composed of lignoceric acid, sphingosine and usually galactose. Such a compound might be expected to produce a positive reaction with the periodic acid leukofuchsin method. This was confirmed by using pure kera<sup>1</sup>sin isolated from human brain. Microscopic localization of this histochemical reaction in sections from three spleens removed from patients with Gaucher's disease was made possible by the fact that the altered carbohydrate remains bound to the insoluble components of kera<sup>1</sup>so. Gaucher cells gave the brilliant rose purple color of a positive reaction. The periodic acid leukofuchsin method has differential diagnostic possibilities for lipid diseases because characteristic foam cells of Niemann-Pick's disease remain colorless.

O P J

## RADIATION AND RADIOACTIVITY

**THE RADIATION SYNDROME** *E. E. Painter and A. M. Bruce* From the Argonne National Laboratory, Chicago, Illinois. *New England J. Med.* 240: 871-876, 1949.

This article is a review of experimental data related to the toxic effect of radiation. The radiation syndrome is divided into the initial shock, the acute period, the subacute period and the chronic period. The authors point out how limited our knowledge is from the problem of how radiation produces cell damage to the interpretation of the many secondary metabolic effects which may be part of the general alarm reaction.

C A F

**THE METABOLISM OF THE RADIOACTIVE ELEMENTS CREATED BY NUCLEAR FISSION** *J. G. Hamilton* From the Crocker Laboratory and Division of Medical Physics (Berkeley), the Divisions of Medicine and Radiology (San Francisco), University of California. *New England J. Med.* 240: 863-870, 1949.

This article deals with the metabolism and tissue localization of products of nuclear fission. To evaluate the potential hazard of these radioactive isotopes, the substances were administered to rats orally by inhalation and by parenteral injection. There are 14 isotopes of importance and their half-life, fission yield, oral absorption, accumulation in the principal organ of retention and elimination are tabulated. Elements such as plutonium deserve special attention due to their skeletal localization and potential danger to the bone marrow. Radioautographs illustrate the osseous and pulmonary localization of some of these elements.

C A F

**THE EFFECT OF ROENTGEN RADIATION ON THE PRODUCTION OF THORACIC DUCT LYMPHOCYTES** *W. N. Valentine, C. G. Cradlock and J. S. Lawrence* From the Departments of Medicine and Radiation Biology, The University of Rochester School of Medicine and Dentistry, Rochester, New York. *Am. J. M. Sc.* 217: 379-382, 1949.

It has been widely recognized that lymphoid tissue is among the most sensitive indicators of damage by roentgen radiation. Further, after large doses of radiation the blood lymphocytes disappear very rapidly from the peripheral blood and are virtually absent in twenty-four hours. In this report changes in the numbers of thoracic duct lymphocytes and rate of flow of thoracic duct lymph in cats receiving a single dose of 1500 r whole body irradiation are recorded. Following this amount of irradiation the number of lymphocytes in thoracic duct lymph decreased rapidly.

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# BONE MARROW NUTRITION IN RELATION TO THE PHAGOCYTTIC ACTIVITY OF BLOOD GRANULOCYTES

By CLARENCE A. MILLS, M D , PH D

PHAGOCYTOSIS has long been recognized as the body's first line of defense against invasion by micro-organisms, with the wandering blood granulocyte playing a particularly important role. Humoral factors which influence the phagocytic activity of these cells have received much attention as basically important elements in the immunity mechanism. Little work has been devoted, however, to the nutritional background of these cells at the time of their production in the bone marrow. Knowledge recently acquired indicates that it is just as important for these fighting units to be well born as it is for the whole individual, that granulocytes produced in the bone marrow during periods of malnutrition or vitamin deficiency remain poorly functioning units throughout their lifetime, while those arising from properly nourished marrow tissue emerge with, and maintain, full phagocytic activity.

In the large group of respiratory infections, and in numerous incidental exposures such as those of burns and wounds, our chief defense against bacterial invasion lies in the basic activity of the phagocytes, unreinforced by the humoral mechanism which may become an important stimulant to phagocytosis only after two to three weeks' exposure. It is therefore essential that we be aware of the conditions promoting or hindering the output of fully active phagocytic granulocytes from the bone marrow.

Development of a quick and relatively simple technic for measuring phagocytic activity of blood granulocytes<sup>1</sup> opened the way for an intensive study of the physiology of these cells in experimental animals kept on synthetic diets.

## METHODS AND RESULTS

A review of the literature concerning phagocytosis and consideration of the various possible techniques convinced us that our best chance to ascertain quantitative differences in phagocytic activity lay in the study of blood leukocytes *in vitro*. The following technic was used:

Under light ether anesthesia 0.5 ml. of blood is withdrawn from the rat's heart into a syringe previously rinsed in heparinized salt solution. This blood is immediately transferred to a paraffined tube of 10 mm. inside diameter and mixed with 0.5 ml. of salt solution containing  $\frac{1}{4}$  mg. of heparin. To this heparinized blood is added 0.1 ml. of a standard bacterial suspension (see below). Air is washed from the tube by a stream of  $O_2$ - $CO_2$  mixture (95%-5%) to maintain a normal blood gas level. The tube is stoppered with a paraffined cork and inverted twice for thorough mixing. It is then placed in a 38°C. water bath and agitated with a lateral motion (360 reversals of direction per minute) for four minutes. A sample is then removed with a small paraffined pipet and a smear is made which is dried and treated with Wright's stain. Four blood samples are usually run together as a group. Careful watch was kept at all times of the temperature of the water bath for changes greater than 1°C. produced distinct alterations in phagocytic activity.

Polymorphonuclear neutrophils of rat blood tend to clump much more readily than do those of man, especially after active ingestion of bacteria has taken place. With the technic just described, however, clumping is rarely observed. The second difficulty—still not completely solved—was a tendency of the

From the Department of Experimental Medicine, University of Cincinnati, Cincinnati.

